UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA
Departamento de Nutrición y Ciencia de los Alimentos

TESIS DOCTORAL

Selección de un probiótico para la erradicación de *Streptococcus agalactiae* durante el embarazo

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Sara Ocaña López

Directores

Juan Miguel Rodríguez Gómez
Virginia Martín Merino
Nivia Cárdenas Cárdenas

Madrid
Ed. electrónica 2019

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Memoria para optar al grado

de Doctor presenta la licenciada Sara Ocaña López

Madrid, 2018

Departamento de Nutrición y Ciencia de los Alimentos

Facultad de Veterinaria

Ciudad Universitaria , s/n. 28040 Madrid
Teléfono: 91 394 3749. Fax: 91 394 37 43

JUAN MIGUEL RODRÍGUEZ GÓMEZ, CATEDRÁTICO DE UNIVERSIDAD, DEL DEPARTAMENTO DE NUTRICIÓN Y CIENCIA DE LOS ALIMENTOS DE LA FACULTAD DE VETERINARIA DE LA UNIVERSIDAD COMPLUTENSE DE MADRID, VIRGINIA MARTÍN MERINO, AYUDANTE DE INVESTIGACIÓN DEL ISCIII CENTRO NACIONAL DE MICROBIOLOGÍA, Y NIVIA CÁRDENAS CÁRDENAS, TÉCNICO SUPERIOR DE INVESTIGACIÓN DE PROBISEARCH,

CERTIFICAN:

Que la Tesis Doctoral titulada "*Selección de un probiótico para la erradicación de Streptococcus agalactiae durante el embarazo*", de la que es autora la Licenciada Sara Ocaña López, ha sido realizada en el Departamento de Nutrición y Ciencia de los Alimentos de la Facultad de Veterinaria de la Universidad Complutense de Madrid, bajo la dirección de los que suscriben, y cumple las condiciones exigidas para optar al título de Doctor.

Madrid, 12 de Noviembre de 2018

Juan Miguel Rodríguez Gómez Virginia Martín Merino Nivia Cárdenas Cárdenas

A los míos, a los que están: mis padres, mi marido, mis hijos, mis tíos, primos y amigos; y a los que me dejaron mis abuelos y tíos.

«Un científico en su laboratorio no es un simple técnico: también es un niño que se enfrenta a fenómenos naturales que lo impresionan como si fueran cuentos de hadas »

Marie Curie

«El científico no tiene por objeto un resultado inmediato. El no espera que sus ideas avanzadas sean fácilmente aceptadas. Su deber es sentar las bases para aquellos que están por venir y señalar el camino»

Nikola Tesla

«Son vanas y están plagadas de errores las ciencias que no han nacido del experimento, madre de toda incertidumbre»

Leonardo da Vinci

Bueno por fin acaba el momento de cerrar una puerta que se abrió hace mucho tiempo, cuando tras acabar Farmacia decidí que quería hacer la Tesis Doctoral. Fueron necesarios tres intentos, pero como suele decirse "a la tercera va la vencida" y ese fue mi caso. Todo esto no hubiera sido posible sin la contribución de personas a las que me gustaría darles las gracias, en primer lugar a mis Directores de Tesis, Juan Miguel Rodríguez, Nivia Cárdenas y Virginia Martin, por vuestro trabajo y ayuda durante estos años. Juan Miguel recuerdo como si fuera ayer la primera charla en la que te oí, y me "enganchaste" por la manera en la que contabas tu trabajo, despertaste en mi un interés inmediato en un tema que hasta ese momento era ajeno a mí, la microbiota, muchas gracias por amar lo que haces, porque sabes transmitir esa inquietud por saber y conocer más, la semilla para la investigación y el conocimiento posterior. Agradecer a CASEN RECORDATI por la financiación que ha permitido la realización de este trabajo.

A mis padres, por siempre apoyarme a lo largo de mi vida, en todas mis decisiones, aunque algunas a veces les duelan. Gracias papá por estar siempre para ayudarme y acompañarme, te quiero muchísimo y me siento muy orgullosa de ti. Mami siempre me has animado a pesar de que a veces ha sido difícil, a buscar tiempo para poder llegar un día a ser Doctora, sin ti yo no lo hubiera conseguido, te amo mamita. Gracias a mi tía Toñi, que está con nosotros, sus sobrinos, siempre dispuesta para todo lo que necesitemos, eres la mejor tía del mundo. A mi compañero de camino, el amor de mi vida, gracias por ayudarme a cumplir mis sueños, los personales y los profesionales. Mis hijitos, Candela, Claudia y José a los que he robado tiempo para poder hacer este proyecto personal, muchas gracias por comprender que a veces mami no podía estar porque estaba trabajando, gracias porque a pesar de no entender me habéis animado. A todos mis amigos, compañeros y familiares que habéis "sufrido" mi Tesis en una u otra manera, muchas gracias por ayudarme, y permitirme llegar a este momento.

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LIST OF ABBREVIATIONS

AMR: Antimicrobial resistance
BA: Biogenic amines
CDC: Centers for Disease Control and Prevention (Estados Unidos)
CFU: Colony-forming units
CLSI: Clinical and Laboratory Standards Institute
CNA: Columbia nalidixic acid medium
CNS: Coagulase-negative staphylococci
CECT: Spanish Type Culture Collection
DAO: Diamino oxidase
DNA: Deoxyribonucleic acid
DSMZ: German Collection of Microorganism and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen)
ECDC: European Center for Disease Control
ECM: Extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
EFSA: European Food Safety Authority
EMBOSS: The European Molecular Biology Open Software Suite
EOS: Early-onset sepsis
FMT: Fecal microbiota transplantation
GAR: *Gardnerella* growth medium
GBS: *S. agalactiae* or group B streptococci
GSH: Glutathione
HIV: Human immunodeficiency virus
HRP: Horseradish peroxidase
IAP: Intrapartum antibiotic prophylaxis
LD: Lethal dose
LOS: Late-Onset Sepsis
MAO: Monoamine oxidase
MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MCK: Mac Conkey medium
MDR: Multidrug (or multiple drug) resistance
MIC: Minimum inhibitory concentration
MLST: Multilocus Sequence Typing
MRS: De Man, Rogosa and Sharpe medium
MRSA: Methicillin-resistant *Staphylococcus aureus*
MVs: Extracellular membrane vesicles
NEC: Necrotizing enterocolitis
NETs: Neutrophil extracellular traps
NF: Nuclear factor
NICU: Neonatal intensive care unit
PCR: Polymerase chain reaction

PFGE: Pulsed-field gel electrophoresis
PRR: Pattern-recognition receptor
QPS: Qualified presumption of safety
RAPD: Random amplification of polymorphic DNA
SDC: Sabouraud dextrose chloramphenicol medium
SEM: Standard error of the mean
SNPs: Single-nucleotide polymorphism
spp.: All individual species within a genus
ST: Sequence Type
TLC: Thin layer chromatography
TMB: Tetramethylbenzidine

I. SUMMARY/RESUMEN

I.SUMMARY

Neonatal sepsis contributes substantially to neonatal morbidity and mortality, and is a major global public health challenge worldwide. According to the age of onset, neonatal sepsis is divided into early-onset sepsis (EOS) and late-onset sepsis (LOS). EOS has been variably defined based on the age at onset, with bacteremia or bacterial meningitis occurring at ≤72 h in infants hospitalized in the neonatal intensive care unit versus <7 days in term infants. EOS reflects transplacental or ascending infections from the maternal genitourinary tract, whereas LOS is associated with the postnatal nosocomial or community environments, with a peak incidence reported to be between days 10 and 22 after birth.

Streptococcus agalactiae (Group B Streptococci, GBS) is one of the microorganisms most frequently involved in severe neonatal EOS cases. Women, men and children of all ages can be asymptomatically colonized with GBS, acting the gastrointestinal tract, vagina and urethra as reservoirs. The maternal colonization rate usually ranges from 12 to 28%. Recto-vaginal GBS screening at week 35-38 of pregnancy, and subsequent intrapartum antibiotic prophylaxis (IAP) to positive mothers, is the most common strategy used to prevent neonatal GBS neonatal infections. However, such strategy does not prevent GBS-related abortions, stillbirths and preterm births, may lead to increasing rates of antibiotic resistance among clinically relevant microorganisms, and has a very negative impact on the acquisition, composition and development of the infant microbiota. GBS vaccines are not available at present and, therefore, there is a need for alternative strategies to avoid GBS colonization during pregnancy. In this context, the general objective of this PhD Thesis was the selection of a safe probiotic strain with the ability to eradicate GBS from the intestinal and genitourinary tracts of pregnant women.

For this purpose, the first partial objective of the Thesis was the study of the vaginal microbiota of non-pregnant and pregnant women, including the assessment of the GBS colonization rates and the preliminary selection of lactobacilli strains from GBS-negative women. A total of 54 fertile healthy women (30 non-pregnant women and 24 pregnant ones) participated in this study. Non-pregnant women provided 4 vaginal exudate samples (days 0, 7, 14 and 21 of their menstrual cycles) while pregnant women provided a single sample collected at week 35-37 of pregnancy. Samples were cultured on a wide variety of broth media and the identification of the isolates was performed by PCR sequencing of the 16S rDNA gene or MALDI-TOF. The percentage of GBS positive women was 19% and 25% among pregnant and non-pregnant women, respectively.

A total of 89 *Lactobacillus* isolates were obtained from the vaginal swabs and, subsequently, submitted to RAPD genotyping to avoid duplication of isolates. Among them, 10 *Lactobacillus salivarius* strains were selected for further characterization on

the basis of the following criteria: (1) absence of *S. agalactiae*, *Gardnella vaginalis*, *Candida spp.*, *Ureaplasma* spp. and *Mycoplasma spp* in the vaginal samples from which the lactobacilli were originally isolated; (2) Qualified Presumption of Safety (QPS) status conceded by EFSA; and (3) ability of the strain to grew rapidly in MRS broth under aerobic conditions ($\geq 1 \times 10^6$ cfu/mL after 16 h at 37ºC).

Initially, an overlay method was used to determine the ability of the lactobacilli strains to inhibit the growth of 12 different *S. agalactiae* strains, 6 isolated from blood or cerebrospinal fluid in clinical cases of neonatal sepsis and the remaining 6 from vaginal samples of pregnant women. Clear inhibition zones (ranging from 2 to 20 mm) were observed around the lactobacilli streaks. In relation to the antimicrobials compounds that may be responsible for such activity, the concentration of L-lactic acid in the supernatants obtained from MRS cultures was similar (~10 mg/mL) for all the lactobacilli. In contrast, D-lactic acid was not detected in the supernatants of the tested strains. All the strains acidified the MRS-broth medium to a final pH of ~4 after 16 h of incubation. No bacteriocin-like activity could be detected against the tested *S. agalactiae* strains and two strains (*L. salivarius* V4II-90 and V7IV-1) were able to produce hydrogen peroxide (~7.4 μg/mL). The capacity of the lactobacilli strains to form large, well defined co-aggregates with *S. agalactiae* was strain-dependent.

Co-cultures with *S. agalactiae* seemed not to affect the growth of any of the *L. salivarius* strains. In contrast, most of the *L. salivarius* strains were able to interfere at a higher or lower degree with the growth of the different *S. agalactiae* strains included in this assay. Among them, *L. salivarius* V4II-90 showed the highest ability to inhibit the growth of *S. agalactiae* since the presence of two of the four *S. agalactiae* strains was not detectable in the co-cultures and the concentration of the other two showed a ~2.5 $\log_{10}$ decrease after an incubation period of only 6 h at 37ºC. No viable streptococci could be detected when the co-cultures were incubated for 24 h.

The lactobacilli strains tested were strongly adhesive to both Caco-2 and HT-29 cells and, in addition, all of them showed adhesion to vaginal epithelial cells. *L. salivarius* V4II-90 globally displayed the highest ability to adhere to both intestinal and vaginal epithelial cells.The lactobacilli strains tested showed a variable ability to adhere to porcine mucin. *L. salivarius* V4II-90 was the strain that showed the highest adherence ability followed by *L. salivarius* V7IV-1. None of the strains were able to degrade gastric mucin *in vitro*.

The viability of the strains after exposition to conditions simulating those found in the gastrointestinal tract varied from ~64% (*L. salivarius* V4II-90) to 30% (*L. salivarius* V3III-1). All the lactobacilli strains were sensitive to most of the antibiotics tested, including those with a special clinical relevance such as, gentamycin, tetracycline, clindamycin, chloramphenicol, and ampicillin, showing MICs equal to, or lower than, the breakpoints defined by EFSA (European Food Safety Authority). All the

strains were resistant to vancomycin and kanamycin, which are intrinsic properties of the *L. salivarius* species. None of the supernatants obtained from the *L. salivarius* strains generated phage-related inhibition halos or plaques of lysis on lawns of any of indicator strains tested in this Thesis. The *L. salivarius* strains neither produced biogenic amines nor harboured the genes required for the biosynthesis of this type of compounds.

Globally, *L. salivarius* V4II-90 was the strain that showed the best results as a candidate for future clinical trials. Subsequently, its potential acute and repeated dose (4 weeks) oral toxicity was evaluated in a rat model; in addition, the potential translocation of this strain to blood and some organs was also investigated in the same animal model.

In the acute (limit test) study, 24 rats (12 males, 12 females) were distributed into two groups of 6 males and 6 females each. Each rat received skim milk (500 µl) orally (control group or Group 1), or a single oral dose of 1×10^{10} colony-forming units (cfu) of *L salivarius* V4II-90 dissolved in 500 µl of skim milk (treated group or Group 2). Animals were checked for clinical signs and mortality twice a day. At the end of a 14 days observation period, the rats were weighed, euthanized and necropsied. No abnormal clinical signs, behavioural changes, body weight changes, macroscopic findings, or organ weight changes were observed. All animals survived the 2-week observation period. There were no statistical differences in body weights among groups. Similarly, no statistically significant differences in body weight gain, food and water consumption were noted. The hematological and clinical chemistry parameters assessed 2 weeks after administration of the strain were not significantly different compared with those of controls. There were no statistical differences in organ weight or tissue: body weight ratios related to the test strain. *L. salivarius* V4II-90 was not associated with any incidence of macroscopic and microscopic changes. Therefore, *L. salivarius* V4II-90 has a low order of acute toxicity and the oral lethal dose (LD_{50}) for male and female rats is higher than 1×10^{10} cfu.

The repeated dose (4 weeks) (limit test) study was conducted in 48 rats (24 males, 24 females) divided in four groups of 6 males and 6 females each (control group or group 3; treated group or group 4; satellite control group or group 5; and satellite treated group or group 6). Rats received a daily dose of either skim milk (groups 3 and 5) or 1×10^{9} cfu of *L. salivarius* V4II-90 dissolved in 500 µl of skim milk (groups 4 and 6) orally once a day over 4 weeks. Animals were checked for clinical signs and mortality twice a day. All rats of the groups 3 and 4 were euthanized and necropsied on day 29. All animals of the satellite groups (groups 5 and 6) were kept a further 14 days without treatment to detect delayed occurrence of toxic effects. All rats of the groups 5 and 6 were euthanized and necropsied on day 42. No mortality was observed. No treatment-related changes in the general condition and external appearance were observed in the treated rats. The development of the animals during the experimental period corresponded to their species and age. There was no significant difference in body weight, body weight gain water and food consumption among groups treated with *L. salivarius* V4II-90 in comparison

to the control groups at any time point of the experimental period. All hematology data were within normal limits and differences between groups were no observed. Clinical chemistry data showed no treatment-related alterations at the end of 4-weeks treatment period. Individual values and group mean values were within the physiologic ranges. Necropsies did not reveal any gross pathological changes or any differences in organ weights in comparison to the corresponding control groups. The no-observed-adverse-effect level in this repeated dose oral toxicity study was the dose tested, i.e. 1×10^9 cfu of *L. salivarius* V4II-90.

In order to determine changes in the antioxidant defense because of the probiotic treatment, liver GSH concentration was determined. No significant differences in liver GSH concentration were observed between control and treated groups. This indicates that treatment with *L. salivarius* V4II-90 did not cause oxidative stress to rats and is consistent with the absence of bacteremia since no lactobacilli could be isolated from blood, liver or spleen of the rats. *L. salivarius* V4II-90 could be isolated from colonic material and vaginal swabs samples of all the treated animals (probiotic groups) at the end of the treatment while it could not be detected in any sample from the placebo groups.

Finally, the efficacy of *L. salivarius* V4II-90 to eradicate GBS from the intestinal and vaginal tracts of pregnant women was evaluated in a human clinical trial. A total of 57 healthy pregnant women (39 rectal and/or vaginal GBS-positive women; 18 rectal and vaginal GBS-negative women at the start of the intervention) participated in this study. Volunteers were distributed into 3 groups. All the volunteers in the probiotic group (n = 25) were GBS-positive and consumed a daily sachet with ~50 mg of freeze-dried probiotic (~9 $\log_{10}$ cfu of *L. salivarius* V4II-90) from week 26 to week 38 of pregnancy. Recto-vaginal GBS screening was performed at 28, 32 and 36-38 weeks. Placebo subgroup 1 (n=14) included GBS-positive women that were going to receive IAP because they had a previous baby that suffered a GBS sepsis. Placebo subgroup 2 (n=18) included GBS-negative women.

All the women of the placebo subgroup 1 remained positive while all the women of the placebo subgroup 2 remained negative at the different sampling points. Women of the probiotic group also tested positive for GBS at 28 weeks, but an increasing number of GBS-negative results appeared in the successive swabs collected until delivery. At 30 weeks, culture of rectal swabs taken from four women of this group rendered a negative result and the number of these samples increased to 18 (72% of the participants) at 38 weeks. Similar results were obtained culturing vaginal swabs obtained from this group, although the proportion of women testing negative for GBS were always slightly higher when analyzing the rectal swabs than in the vaginal ones. In this group, the mean value for *S. agalactiae* counts decreased significantly with the administration time of *L. salivarius* V4II-90, from a mean value of 5.14 cfu/mL at 26 weeks (n=25) to 3.80 cfu/mL at 38 weeks (n=9). No adverse effects arising from the intake of *L. salivarius* V4II-90

were reported by any of the women that participated in this study. Therefore, the clinical trial indicates that *L. salivarius* V4II-90 is a safe and efficient method to reduce the number of GBS-positive women during pregnancy and, therefore, to significantly decrease the number of pregnant women receiving IAP during delivery.

I. RESUMEN

La sepsis neonatal contribuye sustancialmente a la morbilidad y la mortalidad neonatal, y constituye un problema de salud pública muy relevante en todo el mundo. De acuerdo con la edad en la que se inician los síntomas, la sepsis neonatal se divide en sepsis de inicio temprano (EOS) y sepsis de inicio tardío (LOS). Tradicionalmente se ha considerado que las EOS refleja infecciones transplacentarias o ascendentes del tracto genitourinario materno mientras que las LOS se asocian con infecciones nosocomiales o adquiridas en la comunidad, con una incidencia máxima entre los días 10 y 22 después del nacimiento.

Streptococcus agalactiae (*Streptococcus* del grupo B, GBS, EGB) es uno de los microorganismos más frecuentemente implicados en casos graves de EOS. Mujeres, hombres y niños de todas las edades pueden estar colonizados de manera asintomática por este microorganismo, que utiliza el tracto gastrointestinal, la vagina y la uretra como principales reservorios. La tasa de colonización materna generalmente oscila entre el 12 y el 28%. El escrutinio recto-vaginal en la semana 35-38 de embarazo, y la subsiguiente aplicación de la profilaxis antibiótica intraparto (IAP) en aquellas madres positivas, es la estrategia más común para prevenir las infecciones neonatales por GBS. Sin embargo, dicha estrategia no previene los abortos y nacimientos prematuros relacionados con el GBS puede conducir a tasas crecientes de resistencia a antibióticos entre microorganismos clínicamente relevantes, y tiene un impacto muy negativo en la adquisición, composición y desarrollo de la microbiota infantil. Las vacunas frente al GBS no están disponibles en la actualidad y, por lo tanto, existe la necesidad de estrategias alternativas para evitar la colonización por GBS durante el embarazo. En este contexto, el objetivo general de esta Tesis Doctoral fue la selección de una cepa probiótica segura con la capacidad para erradicar el GBS de los tractos intestinal y genitourinario de las mujeres embarazadas.

Para ello, el primer objetivo parcial de la Tesis fue el estudio de la microbiota vaginal de mujeres embarazadas y no embarazadas, incluyendo la evaluación de las tasas de colonización por GBS y la selección preliminar de cepas de lactobacilos a partir de las muestras de aquellas mujeres GBS-negativas. Un total de 54 mujeres fértiles sanas (30 mujeres no embarazadas y 24 embarazadas) participaron en este estudio. Las mujeres no embarazadas proporcionaron 4 muestras de exudado vaginal (días 0, 7, 14 y 21 de sus ciclos menstruales) mientras que las mujeres embarazadas proporcionaron una sola muestra recogida entre las semanas 35-37 de embarazo. Las muestras se cultivaron en una amplia variedad de medios de cultivo y la identificación de los aislados se realizó por secuenciación mediante PCR del gen 16S rDNA o por MALDI-TOF. El porcentaje de mujeres GBS-positivas fue del 19 y del 25% de las mujeres embarazadas y no embarazadas, respectivamente.

Un total de 89 aislados pertenecientes al género *Lactobacillus* fueron aislaron a partir de las muestras vaginales y, posteriormente, se sometieron a un genotipado mediante RAPD para evitar la duplicación de aislados. Entre ellos, se seleccionaron 10 cepas de *Lactobacillus salivarius* para su posterior caracterización sobre la base de los siguientes criterios: (1) ausencia de *S. agalactiae*, *Gardnella vaginalis*, *Candida* spp., *Ureaplasma* spp. y *Mycoplasma* spp en las muestras vaginales a partir de las cuales se habían aislado los lactobacilos; (2) presunción cualificada de seguridad (QPS; EFSA); y (3) la capacidad de la cepa para crecer rápidamente en caldo MRS bajo condiciones aeróbicas (~1×10^6 ufc/ml después de 16 h a 37ºC).

Seguidamente, se determinó la capacidad de los lactobacilos seleccionados para inhibir el crecimiento de 12 cepas de *S. agalactiae*, 6 aisladas de sangre o fluido cerebroespinal en casos clínicos de sepsis neonatal y las 6 restantes de muestras vaginales de mujeres embarazadas. Todas los lactobacilos mostraron capacidad para inhibir el crecimiento de las cepas de *S. agalactiae*. En relación con los compuestos antimicrobianos que pudieran ser responsables de dicha actividad, la concentración de ácido L-láctico en los sobrenadantes obtenidos a partir de los cultivos en MRS fue similar (~10 mg/ml) para todos los lactobacilos. Por el contrario, no se detectó ácido D-Láctico en los sobrenadantes de las cepas probadas. Todas las cepas acidificaron el medio MRS hasta un pH final de ~4 tras 16 h de incubación. Ninguna cepa mostró actividad bacteriocinogénica frente a las cepas de *S. agalactiae* mientras que dos de ellas (*L. salivarius* V4II-90 y V7IV-1) produjeron peróxido de hidrógeno (~7,4 μg/ml). La capacidad de las cepas de lactobacilos para formar coagregados grandes y bien definidos con cepas de *S. agalactiae* fue variable dependiendo de cada cepa.

Los co-cultivos con *S. agalactiae* no afectaron al crecimiento de ninguna de las cepas de *L. salivarius*. Por el contrario, la mayoría de las cepas de *L. salivarius* interfirieron, en mayor o menor grado, el crecimiento de las diferentes cepas de *S. agalactiae* incluidas en este ensayo. Entre ellos, *L. salivarius* V4II-90 mostró la mayor capacidad para inhibir el crecimiento de *S. agalactiae* ya que la presencia de dos de las cuatro cepas de *S. agalactiae* no fue detectable en los co-cultivos tras 6 h de incubación a 37ºC mientras que la concentración de las otras dos mostró una disminución notable (~2.5 $\log_{10}$) en el mismo periodo. En ningún caso se pudieron detectar estreptococos viables cuando los co-cultivos se incubaron durante 24 h.

Las cepas de lactobacilos fueron fuertemente adhesivas a células Caco-2 y HT-29 y, además, todas mostraron adhesión a células epiteliales vaginales. *L. salivarius* V4II-90 fue la cepa que mostró mayor capacidad para adherirse a las células epiteliales, tanto intestinales como vaginales. Las cepas de lactobacilos mostraron una capacidad variable para adherirse a la mucina porcina siendo nuevamente *L. salivarius* V4II-90 la cepa que mostró la mayor capacidad de adherencia. Ninguna de las cepas fue capaz de degradar mucina gástrica *in vitro*.

La viabilidad de las cepas después de la exposición a condiciones que simulan las del tracto gastrointestinal osciló entre un 64 (*L. salivarius* V4II-90) y un 30% (*L. salivarius* V3III-1). Todas las cepas fueron sensibles a la mayoría de los antibióticos evaluados, incluidos aquellos clínicamente relevantes como gentamicina, tetraciclina, clindamicina, cloranfenicol y ampicilina, y mostraron CMIs iguales o inferiores a los puntos de corte definidos por la EFSA. Todas las cepas fueron resistentes a vancomicina y kanamicina, propiedades intrínsecas de la especie *L. salivarius*. Ninguna de las cepas parecía poseer fagos líticos y ninguna de ellas produjo aminas biogénicas ni albergaba los genes requeridos para la biosíntesis de este tipo de compuestos.

Globalmente, *L. salivarius* V4II-90 fue la cepa que mostró los mejores resultados como candidata para futuros ensayos clínicos. Posteriormente, se evaluó su posible toxicidad oral aguda y crónica (4 semanas) en un modelo de rata; además, también se investigó la posible translocación de esta en ese mismo modelo.

En el estudio de toxicidad aguda, se distribuyeron 24 ratas (12 machos, 12 hembras) en dos grupos de 6 machos y 6 hembras cada uno. Cada rata recibió leche desnatada (500 µl) por vía oral (grupo control o Grupo 1), o una dosis oral única de 1×10^{10} ufc de *L salivarius* V4II-90 disuelto en 500 µl de leche desnatada (grupo tratado o grupo 2). La posible presencia de signos clínicos y mortalidad se evaluó dos veces al día. Al final de un período de observación de 14 días, las ratas fueron pesadas, sacrificadas mediante eutanasia y sometidas a necropsia. No se observaron signos clínicos anormales, cambios en el comportamiento, cambios en el peso corporal, hallazgos macroscópicos anómalos o cambios en el peso de los órganos. Todos los animales sobrevivieron el período de observación de 2 semanas. No hubo diferencias en el peso corporal entre los dos grupos. Del mismo modo, no se observaron diferencias estadísticamente significativas en el consumo de alimentos y de agua. Los parámetros hematológicos y bioquímicos evaluados 2 semanas después de la administración de la cepa no fueron significativamente diferentes en comparación con los de los controles. La administración de *L. salivarius* V4II-90 no se asoció con cambios macroscópicos o microscópicos en los órganos de los animales. Por lo tanto, *L. salivarius* V4II-90 tiene una toxicidad aguda muy baja y la dosis letal oral (DL_{50}) para ratas es superior a 1×10^{10} cfu.

El estudio de toxicidad a dosis repetidas (4 semanas) se realizó con 48 ratas (24 machos, 24 hembras) divididas en cuatro grupos de 6 machos y 6 hembras cada uno (grupo control o grupo 3; grupo tratado o grupo 4; grupo control satélites o grupo 5, y grupo tratado satélite o grupo 6). Las ratas recibieron una dosis diaria de leche desnatada (grupos 3 y 5) o 1×10^{9} ufc de *L. salivarius* V4II-90 disueltos en 500 µl de leche desnatada (grupos 4 y 6) por vía oral una vez al día durante 4 semanas. Todas las ratas de los grupos 3 y 4 fueron sacrificadas y necropsiadas el día 29. Todos los animales de los grupos satélites (grupos 5 y 6) se mantuvieron otros 14 días sin tratamiento para detectar la aparición tardía de posibles efectos tóxicos. No se observaron cambios

relacionados con el tratamiento en el estado general o la apariencia externa en las ratas tratadas. El desarrollo de los animales durante el período experimental correspondió a su especie y edad. No hubo diferencias significativas en el peso corporal, el aumento de peso corporal, el consumo de agua y el consumo de alimentos entre los grupos tratados con *L. salivarius* V4II-90 en comparación con los grupos control. Todos los datos hematológicos y de bioquímica clínica se encontraron dentro de los límites normales al final del período de tratamiento de 4 semanas. Las necropsias no revelaron ningún cambio patológico ni ninguna diferencia en el peso de los órganos en comparación con los grupos control correspondientes. El nivel de efecto adverso no observado en este estudio de toxicidad oral a dosis repetidas fue a la dosis probada, es decir, 1×10^9 cfu de *L. salivarius* V4II-90.

Tampoco se observaron diferencias significativas en la concentración de GSH hepático entre los grupos control y los tratados. Esto indica que el tratamiento con *L. salivarius* V4II-90 no causó estrés oxidativo a las ratas y es consistente con la ausencia de bacteriemia ya que no se pudieron aislar lactobacilos de la sangre, hígado o bazo de las ratas tratadas. *L. salivarius* V4II-90 se pudo aislar de muestras vaginales y rectales de todos los animales tratados al final del tratamiento, mientras que no se pudo detectar en ninguna muestra de los grupos control.

Finalmente, se evaluó la eficacia de *L. salivarius* V4II-90 para erradicar GBS del tracto intestinal y vaginal de mujeres embarazadas sanas. Un total de 57 mujeres embarazadas sanas (39 GBS-positivas y 18 GBS-negativas al inicio de la intervención) participaron en el estudio. Las voluntarias fueron distribuidas en 3 grupos. Todas las voluntarias del grupo probiótico (n = 25) eran GBS-positivas y consumieron un sobre diario con ~50 mg de probiótico liofilizado (~9 $\log_{10}$ cfu de *L. salivarius* V4II-90) desde la semana 26 hasta la semana 38 de embarazo. El cribado de GBS recto-vaginal se realizó a las 28, 32 y 36-38 semanas. El subgrupo placebo 1 (n = 14) incluyó mujeres con GBS-positivas que iban a recibir IAP durante el parto debido a que tuvieron un niño anterior que sufrió una sepsis neonatal por GBS. El subgrupo placebo 2 (n = 18) incluyó a las mujeres GBS-negativas.

Todas las mujeres del subgrupo placebo 1 siguieron siendo GBS-positivas y todas las mujeres del subgrupo placebo 2 GBS-negativas en todos los puntos de muestreo. Las mujeres del grupo probiótico también seguían siendo GBS-positivas a las 28 semanas pero, a partir de ese momento, el número de GBS-negativas fue creciendo hasta la semana 38, en la que so tomó la última muestra. Así, cuatro mujeres de este grupo fueron negativas en las muestras rectales a las 30 semanas y su número aumentó hasta 18 (72% de las participantes) a las 38 semanas. Resultados similares se observaron con las muestras vaginales. En el grupo probiótico, los recuentos de *S. agalactiae* también disminuyeron significativamente durante el periodo de administración de *L. salivarius* V4II-90, desde un valor medio de 5,14 ufc/ml a las 26 semanas (n=25) hasta 3,80 ufc/ml a las 38 semanas (n=9) Ninguna mujer sufrió efectos adversos derivados de

la ingesta de *L. salivarius* V4II-90. En conclusión, el ensayo clínico indicó que la administración de dicha cepa es un método seguro y eficaz para reducir el número de mujeres GBS-positivas durante el embarazo y, por lo tanto, para disminuir significativamente el número de mujeres embarazadas expuestas a la IAP durante el parto.

II. INTRODUCTION

II.1. NEONATAL SEPSIS

Neonatal sepsis is a systemic infection occurring in infants at ≤28 days of life. The World Health Organization estimates that 1 million deaths per year (10% of all under-five mortality) are due to neonatal sepsis and that 42% of these deaths occur in the first week of life (Lawn et al., 2005). There are wide disparities in neonatal care between high- and low-income countries. In high-income countries the major concerns are the high nosocomial infection rates associated to the increasing numbers of extremely preterm infants and the potential long term sequela while, in low-income ones, the more pressing issues are the high proportion of deliveries in unclean environments and the lack of suitable medical support, two facts predisposing to high rates of sepsis and death. Other risk factors for neonatal sepsis include prolonged rupture of membranes, preterm labor, low birth weight, underlying diseases, failure of early feeding with human colostrum and milk, and genetic factors, such as the polymorphism in immunity-associated genes (Schuchat et al., 2000).

Neonatal sepsis contributes substantially to neonatal morbidity and mortality, and is a major global public health challenge worldwide (Qazi and Stoll, 2009). According to the age of onset, neonatal sepsis is divided into early-onset sepsis (EOS) and late-onset sepsis (LOS). EOS has been variably defined based on the age at onset, with bacteremia or bacterial meningitis occurring at ≤72 h in infants hospitalized in neonatal intensive care unit (NICU) versus <7 days in term infants (Vergnano et al., 2011). Traditionally, EOS has been considered to reflect transplacental or ascending infections from the maternal genitourinary tract, whereas LOS has been generally associated with the postnatal nosocomial or community environment, with a peak incidence between days 10 and 22 after birth (van den Hoogen et al., 2010).

Since the early 1980s, epidemiological studies have observed a general reduction in EOS, probably due to advances in obstetric and neonatal care. In contrast, the incidence of LOS has increased in parallel with the higher survival rates of premature infants, especially of those with short gestational ages and/or extremely low or very low birth weights in developed countries, indicating the role of hospitalization and life-sustaining medical devices in the pathogenesis of neonatal LOS. The characteristics of the microorganisms causing neonatal sepsis are of primary importance in guiding clinical practice, and strategies to prevent and treat them may, in turn, influence the pattern of pathogens involved in such infections. In fact, the etiology of neonatal sepsis varies with geographical location and changes over time (Ohlsson et al., 1986).

The microorganisms most frequently involved in EOS, taking in account both term and preterm infants together, are *Streptococcus agalactiae* (group B streptococci, GBS) and *Escherichia coli*, which account for approximately 70% of infections (Simonsen et al., 2014). Additional pathogens to consider, which account for the remaining minority of cases, are other streptococci (most commonly viridans group streptococci but also

Streptococcus pneumoniae), *Staphylococcus aureus*, *Enterococcus* spp., other Gram-negative enteric bacilli (such as *Enterobacter* spp.), *Haemophilus* spp., and *Listeria monocytogenes* (Simonsen et al., 2014). However, when only preterm infants are considered, the burden of disease attributable to *E. coli* and other Gram-negative rods increases, making Gram-negative sepsis the most common etiology of EOS in this population (Hornik et al., 2012).

In relation to LOS, coagulase-negative staphylococci (CNS) have emerged as the predominant etiological agents, accounting for 53–78% of LOS in industrialized countries and 35–47% in some developing regions (Hammoud et al., 2012; Tsai et al., 2014). Globally, CNS are not so virulent as Gram-negative bacteria and fungi, which partly explains the lower rates of short-term infectious complications and mortality associated with CNS sepsis (Tsai et al., 2014). However, the risk of neurodevelopment sequelae, such as cognitive and psychomotor impairment, cerebral palsy, and vision impairment was independent of the type of pathogen, indicating that CNS are capable to exert a long-term detrimental effect on the host, particularly on the most immature infants with a birth weight <1,000 g (Stoll et al., 2004). Recent data shows that CNS, predominantly *Staphylococcus epidermidis*, is highly variable in genetic background and can acquire pathogenic determinants, such as the ability to form biofilms and antimicrobial resistance in order to adapt to the nosocomial environment (Lepainteur et al., 2013; Dong and Speer, 2014). Gram-negative bacilli responsible for neonatal LOS are mainly *E. coli*, *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp. and *Pseudomonas* spp. (Dong and Speer, 2014). In addition, fungi (especially *Candida* spp.) are reported to be major LOS pathogens in some regions, particularly in extremely low weight preterm neonates (Leal et al., 2012).

The distribution pattern of causative pathogens varies across regions and may change over time within the same hospital due to demographic characteristics of patients, host and environmental microbiota and the policy of feeding and antibiotic use. It should be noted that the wide (and often empirical) use of broad-spectrum antibiotics in the past decades has contributed to an increasing incidence of multidrug (MDR)-resistant Gram-negative bacilli, which account for approximately 20% of bacteremia cases, and are associated with a 2.8-fold increase in neonatal mortality rate when compared to cases of sepsis caused by non-MDR strains (Tsai et al., 2014).

Globally, preterm birth is a significant contributor to neonatal death. Every year, approximately 6 000 000 births are preterm, and more than 500 000 neonates die due to prematurity, accounting for 44% of all deaths under the age of 5 years (Liu et al., 2015; Mokdad et al., 2016). The majority of early preterm births are due to microbial infection (Romero et al., 2014), and approximately 10% are attributable to GBS (Hitti et al., 2001; DiGiulio et al., 2008; Han et al., 2009).

II.2. *Streptococcus agalactiae*

II.2.1. Historical perspective and general characteristics

Streptococcus agalactiae (also known as group B streptococcus or GBS) is a Gram-positive coccus with a tendency to form chains. It is a beta-hemolytic, catalase-negative, and facultative anaerobe bacteria. In general, GBS is a harmless commensal bacterium being part of the human microbiota colonizing the gastrointestinal and genitourinary tract of up to 30% of healthy human adults (asymptomatic carriers). Nevertheless, under certain circumstances, GBS can cause severe invasive infections.

S. agalactiae is the species designation for streptococci belonging to group B of the Lancefield classification. GBS is surrounded by a capsule composed of exopolysaccharides. The species is subclassified into ten serotypes (Ia, Ib, II–IX) depending on the immunologic reactivity of their polysaccharide capsule. This is why the plural term group B streptococci (referring to the serotypes) and the singular term group B streptococcus (referring to the single species) are both commonly encountered.

GBS grows readily on blood agar plates as colonies surrounded by a narrow zone of β-hemolysis although there are non-hemolytic strains. GBS is characterized by the presence in the cell wall of the antigen group B of Lancefield classification that can be detected directly in intact bacteria using latex agglutination tests. Before the availability of PCR and MALDI-TOF as the main techniques for bacterial identification, the CAMP test was also another important test for identification of GBS. The CAMP factor produced by GBS acts synergistically with the staphylococcal β-hemolysin inducing enhanced hemolysis of sheep or bovine erythrocytes. GBS is also able to hydrolyze Hippurate and this test was also used to identify presumptively GBS. Hemolytic GBS strains produce an orange-brick-red non-isoprenoid polyene pigment (granadaene) when cultivated on Granada medium that allows its straightforward identification. The GBS properties that are relevant for laboratory identification of GBS have been reviewed recently (Rosa-Fraile and Spellerberg, 2017).

GBS was first identified in 1887 as a cause of bovine mastitis (Nocard and Mollereau, 1887). Later it was isolated from the human vagina of asymptomatic carriers (Lancefield and Hare, 1935) and associated with cases of human disease (Fry, 1938). The first report of GBS sepsis in a neonate did not appear until 1964 (Eickhoff et al., 1964). Since the 1970s, GBS is considered one of the main risk factors for preterm birth (Allen et al., 1999; Lawn et al., 2010) and one of the most common causes of neonatal infectious morbidity and mortality in Europe, North America and Australia (McCracken, 1973; Bergqvist, 1974; Lloyd and Reid, 1976; Schröder and Paust, 1979; Allardice et al., 1982; Vesikari et al., 1989; Fliegner and Garland, 1990; Stoll et al., 2011; Le Doare and Heath, 2013).

GBS serotypes Ia, II, III and V are responsible for early onset episodes caused by this species (Harrison et al., 1998; Zaleznik et al., 2000; Weisner et al., 2004); In contrast, late onset disease is caused predominantly by serotype III (Le Doare and Heath, 2013)]. Cases occurring later in infancy than 90 days are also described, but rare, and generally associated with extreme prematurity. In 1973, Baker and Barrett demonstrated that whilst all GBS serotypes were capable of causing neonatal infection, type III isolates were significantly increased amongst infants with GBS meningitis. GBS multilocus sequence typing (MLST) using a global collection of isolates demonstrated that capsular serotype does not strictly follow sequence type (ST) and that a single, bovine-derived ST (ST-17), appears to be overrepresented in neonatal disease (Bisharat et al. 2004). Further work from the USA and the UK determined that ST-17 in types II and III GBS are closely related to ancestral bovine isolates (Jones et al., 2006; Bohnsack et al., 2008). This sequence type appears to be associated with neonatal disease, irrespective of the capsular serotype, but not with adult disease (Jones et al., 2006).

In the USA and Europe, invasive GBS-serotypes are predominantly Ia, Ib, II, III and V (Baker and Kasper, 1976; Baker et al., 1978; Lin et al., 2001; Lin et al., 2004) whilst a Gambian study reported serotype-V predominance (Suara et al., 1998). The recent global review of invasive isolates showed that serotype III was the most frequently identified serotype in all regions with available data (48.9%), followed by serotypes Ia (22.9%), V (9.1%), Ib (7.0%) and II (6.2%). There was little change in ST distribution over the last 30 years (Edmon et al., 2012). There was however, little data from low/middle income countries.

II.2.2. GBS colonization and mother-to-infant transmission

Women, men and children of all ages in both developed and developing countries can be colonized with GBS without having any symptoms. The gastrointestinal tract, vagina and urethra serve as reservoirs for GBS. In other words, *S. agalactiae* is a pathobiont that is often part of the normal microbiota found in the gastrointestinal and genitourinary tracts of healthy women (Katz et al., 1993; Blumberg et al., 1996; Wessels et al., 1997; Verani et al., 2010; Edwards et al., 2011).

Pioneer studies showed that the maternal colonization rate was, approximately, 18% (ranging from 12 to 28%), independently of the countries or socio-economical status of the screened women (Ohlsson, 1992; Stoll and Schuchat, 1998). A systematic review on the prevalence of maternal GBS colonization in European countries between 1996 and 2006 revealed GBS vaginal colonization rates ranging from 6.5% to 36%, with one-third of the studies reporting rates of 20% or greater. The carriage rates in Southern Europe oscillated from 6.5 to 32% (Barcaite et al., 2008) while they have been reported to oscillate from 12 to 20% in Spain (Alos et al., 2012).

A recent systematic review and meta-analyses found that adjusted estimate for maternal GBS colonization worldwide was 18% (95% confidence interval [CI], 17%–

19%), with regional variation (11%–35%), and lower prevalence in Southern Asia (12.5% [95% CI, 10%–15%]) and Eastern Asia (11% [95% CI, 10%–12%]) (Russell et al., 2017). Bacterial serotypes I–V account for 98% of identified colonizing GBS isolates worldwide. Serotype III, associated with invasive disease, accounts for 25% (95% CI, 23%–28%), but is less frequent in some South American and Asian countries (Russell et al., 2017). Serotypes VI–IX are more common in Asia GBS colonizes pregnant women worldwide, but prevalence and serotype distribution vary, even after adjusting for laboratory methods. Lower GBS maternal colonization prevalence, with less serotype III, may help to explain lower GBS disease incidence in regions such as Asia (Russell et al., 2017).

The transmission rate for GBS colonization from mother to infant varied from 35% (England) to 69% (Brazil). However, only 1-2% of the neonates born from non-treated GBS-positive women develop GBS sepsis (Baker and Edwards, 1995). Recently, it has been estimated that, of 140 million live births in 2015, there were 21.3 million (UR, 16.4–27.0 million) infants exposed to maternal GBS colonization at delivery (Seale et al., 2017).

Women who are vaginally colonized during pregnancy are at risk for ascending infection or transmission of GBS to the newborn during delivery. Ascending infection is a widely accepted route by which vaginal bacteria move from the vagina, through the cervix, and into the uterus and penetrate gestational tissues (Figure 1). Once GBS has invaded the amniotic cavity, or come into contact with the placenta, there is the potential for chorioamnionitis or inflammation of the placental membranes that is frequently associated with preterm births and stillbirths (Romero, 2014) (Figure 2). The bacterial and host determinants that promote GBS vaginal colonization, ascending infection, and adverse perinatal outcomes are poorly understood (see next section).

In addition to asymptomatic carriage, GBS can also cause maternal infections, including urinary tract infections, vulvovaginitis, cervical dysplasia, endometritis, intra-amniotic infection and wound infections (Figure 2). Recently, the first review assessing invasive maternal GBS disease found an incidence of 0.38 (95% CI, .28–.48) per 1000 pregnancies and 0.23 (95% CI, .09–.37) per 1000 maternities in high-income contexts (Hall et al., 2017). This maternal incidence is lower than the incidence of neonatal GBS disease (0.42 [95% CI, .30–.54]) in developed countries (Madrid et al., 2017), but it is likely to be an underestimate due to underreporting and/or low case ascertainment. Anyway, the risk of mortality and morbidity for women with maternal GBS disease appears low in the developed region (case fatality risk, 0.19% [95% CI, –0.25% to 0.62%]) (Hall et al., 2017).

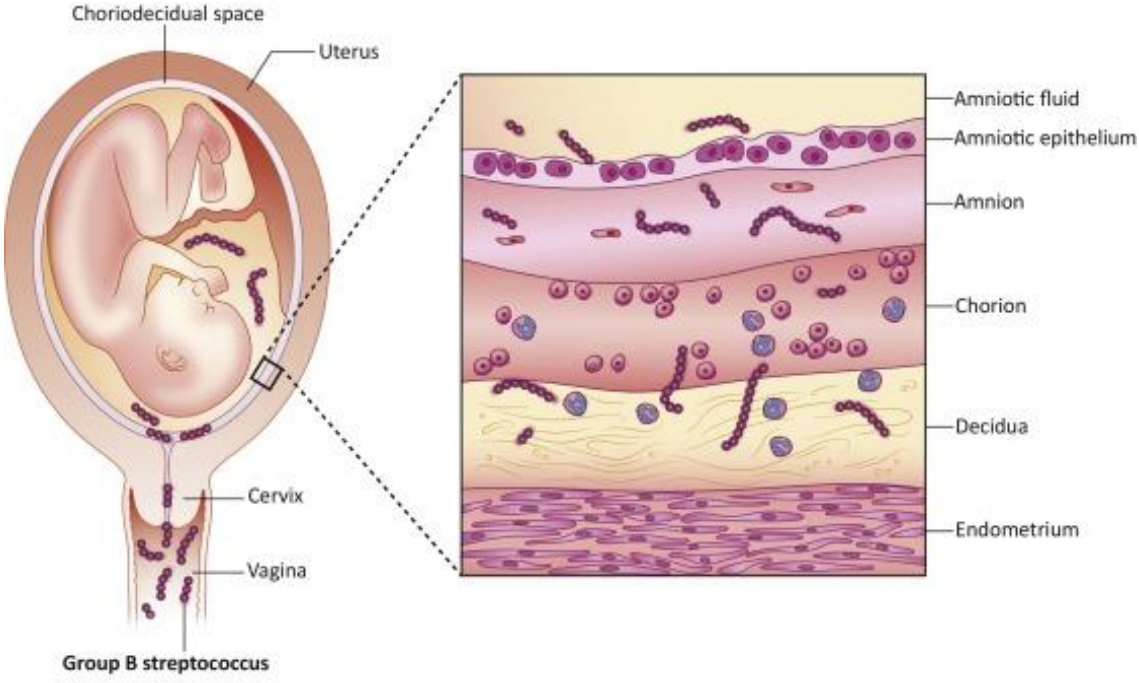


Figure 1. Ascending GBS infection. GBS vaginal colonization increases the risk of ascending infection during pregnancy. Ascending GBS infection during pregnancy involves bacterial trafficking from the vagina, ultimately leading to bacterial invasion of placental membranes (chorion and amnion), the amniotic cavity, and the fetus. GBS expresses a number of virulence factors that promote vaginal colonization, adhesion and invasion of host cells, and for either activation or suppression of inflammatory responses. These factors increase the risk of ascending infection, fetal injury, or preterm birth. Source: Vornhagen et al. (2017).

II.2.3. Risk factors for maternal GBS colonization, transmission of GBS from mother to baby, neonatal GBS colonization or GBS neonatal disease

Numerous maternal, obstetric and neonatal factors have been associated with or identified as risk factors for maternal GBS colonization, transmission of GBS from mother to baby, neonatal GBS colonization or neonatal disease with GBS (both EO and LO). Although some of these have provided useful insights into the pathophysiology of perinatal GBS disease, many of them are interrelated, studies have produced contradictory results and relatively few factors are sufficiently robust or clinically relevant enough to guide prevention strategies.

The gastrointestinal tract is the primary reservoir of GBS and the source of vaginal colonization in women. Local hygiene or sexual practices can increase the risk for vaginal colonization. Other factors associated with maternal colonization include ethnicity (women of black race), use of tampons or intrauterine devices, obesity, absence of lactobacilli in the gastrointestinal flora and preterm delivery (Parry et al., 1998; Schuchat, 1999; Oddie and Embleton, 2002). GBS bacteriuria during pregnancy is

associated with heavy colonization, a further risk factor for perinatal transmission (Persson et al., 1985; Kessous et al., 2012). Mothers with GBS bacteriuria demonstrate a higher incidence of adverse obstetric outcomes: habitual abortion, intrauterine growth restriction, preterm labour, chorioamnionitis and premature rupture of membranes (Kessous et al., 2012). Factors associated with an increased risk of neonatal colonization include maternal colonization, male sex, black race, prolonged rupture of membranes, prematurity, low levels of maternal anti-GBS antibodies and intrapartum fever.

II.2.4. Neonatal disease by GBS

GBS disease is not restricted to newborns, but its greatest impact, both in terms of severity and incidence, is in the neonatal period and up to the first 90 days of life. Early onset (EO) GBS disease is usually defined as infection presenting in the first six days of life and accounts for approximately 60–70% of all GBS disease. As stated above, GBS serotypes Ia, II, III and V are responsible for most EO disease (Harrison et al., 1998; Zaleznik et al., 2000; Weisner et al., 2004). Maternal carriage of GBS in the gastrointestinal and/or genital tracts is a pre-requisite for EO disease, vertical transmission occurring during or just prior to birth. In developed countries an estimated 20–30% of pregnant women are colonized with GBS (Bergeron et al., 2000; Jones et al., 2006b), approximately 50% of their babies become colonized and 1% progress to develop invasive disease. Disease may occur rapidly; signs are evident at birth or within 12 h in over 90% of cases (98% within the first 12 h) and presentation is typically with pneumonia or sepsis (Heath et al., 2004). In contrast, late-onset (LO) disease is caused predominantly by serotype III, is acquired perinatally, nosocomially or from community sources, and in up to 50% of cases presents with meningitis (Easmon et al., 1981; Hastings et al., 1981; Weisner et al., 2004; Heath et al., 2004). Cases occurring later in infancy than 90 days are also described, but rare, and generally associated with extreme prematurity (Schuchat et al., 1990; Zangwill et al., 1992; Lin et al., 2003; Verani et al., 2010; Stoll et al., 2011).

The burden of neonatal GBS disease can be defined in terms of its incidence, morbidity and mortality. Multistate, population-based, active surveillance in the USA in 1990 identified an EO disease rate of 1.4 per 1000 live births and a LO rate of 0.4 per 1000 births (Zangwill et al., 1992). This equated to 7600 cases and 310 deaths per year in the USA. Both EO and LO GBS disease occurred at significantly higher rates amongst African-American compared to Caucasian infants, as well as amongst low-birthweight or preterm infants (Schuchat et al., 1990; Zangwill et al., 1992). In the UK, a multi-center study carried out between 1977 and 1978 recorded an incidence of invasive GBS disease in infants < 2 months of age of 0.3 cases per 1000 live births (Stringer et al., 1981). Through the late 1990s studies from different centers in England reported incidence figures ranging from 0.6 to 1.2 cases per 1000 live births (Moses et al., 1998; Bignardi et al., 1999; Beardsall et al., 2000). In 2000–2001, national surveillance was enhanced; the overall incidence was 0.72 per 1000 live births (95% confidence interval

0.66–0.78), 0.47 per 1000 (0.42–0.52) for EO disease and 0.25 per 1000 (0.21–0.28) for LO disease (Heath et al., 2004). Regional variation was marked; the incidence in Scotland was 0.42 per 1000 whilst in Northern Ireland it was 0.9 per 1000 live births.

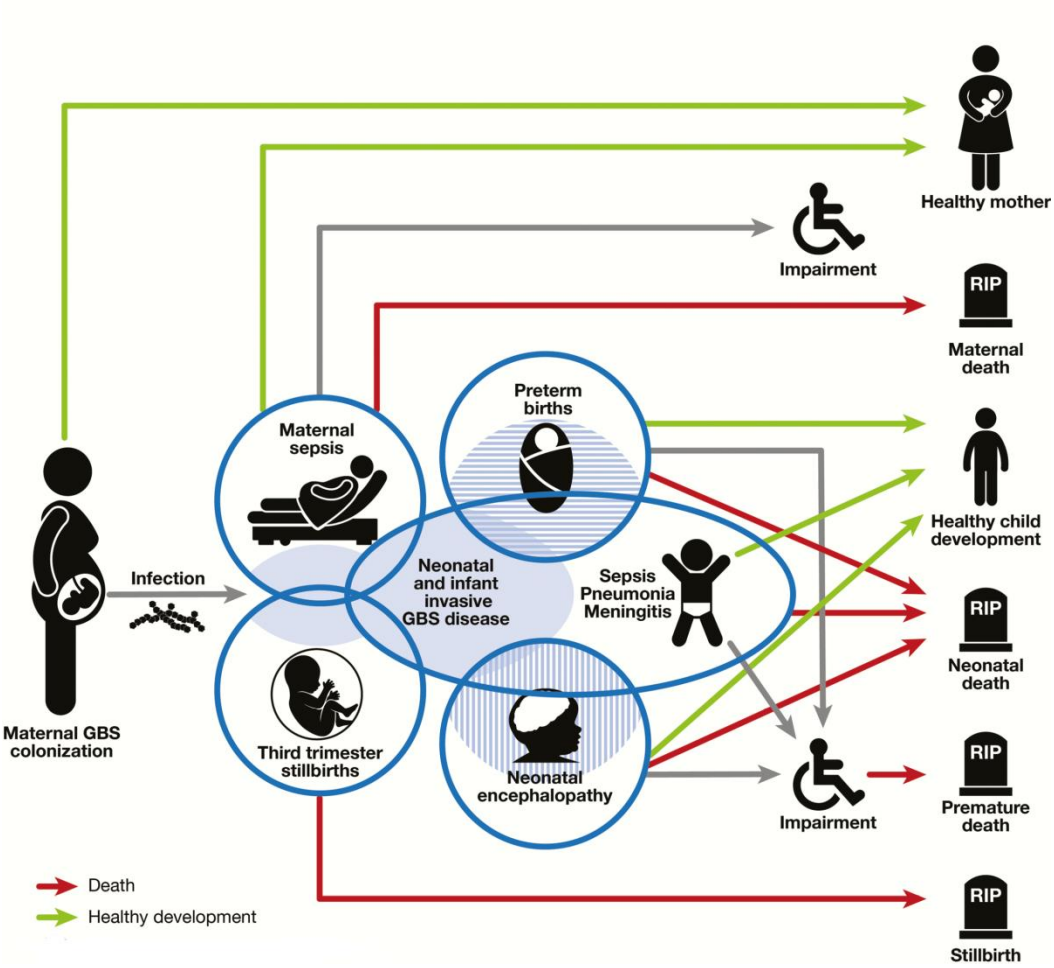


Figure 2. Maternal, fetal and infant disease oucomes after a GBS infection; source: Lawn et al. (2017).

As with other studies, infants of low birthweight (<1500 g) were shown to have the highest rates of GBS disease (Heath et al., 2004). National population-based surveillance studies have defined the incidence <3 months of age in a number of developed countries including Portugal (2001–2005, 0.54/1000), Finland (1985–1994, 0.76/1000) and Germany (2001–2003, 0.47/1000) (Le Doare and Heath, 2013).

Hospital based surveillance studies (which may be biased towards higher risk pregnancies) have generally yielded higher incidences such as in Australasia (EO GBS (<48 h of age): 2/1000 in 1991–1993, 1.3/1000 in 1993–1995, 0.5/1000 in 1995–1997) (Isaacs and Royle, 1999) and Spain (EO GBS (<3 days of age): 1.1/1000, 1996–1997; 0.7/1000, 2000–2001) (López Sastre et al., 2005). Edmonds et al. recently conducted a systematic review to define the current global incidence of GBS disease in infants under

3 months (Edmon et al., 2012). The analysis was confined to data since the year 2000 and estimated an overall incidence of 0.53 (0.44–0.62) in the European region, 0.67 (range 0.54–0.80) in the Americas and 0.15 (range−0.03 to 0.07) in Australasia.

GBS disease is also associated with significant morbidity and mortality. GBS meningitis, in particular, results in long term neurodevelopmental impairment. Bedford et al. (2001) described 5-year neurodevelopmental outcomes following an acute episode of neonatal meningitis in 98 children, of whom 13% had severe, 17% moderate and 18% mild disability at 5 years. Overall 50% of those with GBS meningitis had neurodevelopmental impairment at 5 years of age. A recent small series from the USA has shown similar rates of sequalae (up to 44% with moderate–severe impairment) (Libster et al., 2012).

Case fatality rates for GBS disease are estimated at between 9% in Oxford (1990–1996) (Moses et al., 1998) and 15% in London (1990–1999) (Mifsud et al., 2004), with a UK national figure of 10% in 2000–2001 (Heath et al., 2004). In the USA between 1993 and 1998 the quoted case fatality rates for EO and LO disease were 4.7% and 2.8% respectively (Schrag et al., 2000). Case fatality rates were significantly higher in premature infants (<33 weeks gestation) 15.2% vs. 6.4% for term infants (>37 weeks gestation), relative risk of 6.7 in EO GBS (21, 39). Other national studies have revealed case fatality rates of 6.6% in Portugal, 4.3% in Germany and 8% in Finland (Le Doare and Heath, 2013). Overall current case fatality rates by region as estimated in the Edmond meta-analysis are 0.07 (range 0.04–0.1) in Europe and 0.11 (0.06–0.16) in the Americas (Edmon et al., 2012).

II.2.5. Mechanisms for GBS infection during pregnancy

It is long known that GBS vaginal colonization during pregnancy is associated with increased rates of neonatal infection (Hoogkamp-Korstanje, et al., 1982), recurrent maternal colonization (Cheng et al., 2008), early-term birth (weeks 37–38, and 6 days of gestation) (Mitchell et al., 2013), preterm birth (weeks 14–36, and 6 days of gestation) (Lawn et al., 2010), and stillbirth (Monari et al., 2013). GBS is thought to be transmitted from person to person via multiple routes, including fecal–oral, sexual, and vertical transmission (Manning et al., 2004). In the same woman, the close proximity of the vagina and rectum likely enables GBS trafficking from intestinal microbiota into the vagina. Once GBS enters the vagina, colonization requires the bacteria to overcome a number of challenges, including: physical barriers created by the mucus and epithelial layers, low environmental pH, antimicrobial peptides, antibodies, microbicidal immune cells, and a vaginal microbiome dominated by lactobacilli.

How a non-motile bacterium, such as GBS, manages to ascend into the uterus while evading host responses is not completely understood. The process of ascending infection is challenging to study, as *in vitro* models are unsuitable, and *in vivo* models are limited in their ability to recapitulate human pregnancy (McDuffie and Gibbs, 1994;

De Clercq et al., 2013). Despite these limitations, a number of animal models, including pregnant mice and non-human primates, have been developed to study the mechanisms of ascending GBS infection (Ancona and Ferrieri, 1979; Adams Waldorf et al., 2011; Randis et al., 2014; Kolar et al., 2015; Vornhagen et al., 2016; Boldenow et al., 2016; Kothary et al., 2017), shedding new light on these complicated processes. While studies using these models have revealed a novel insight into the role of virulence factors that contribute to ascending infection, more research is needed to fully understand the process of ascending GBS infection and adverse neonatal outcomes.

The host immune response evoked in the placenta in response to GBS infection is a key determinant of perinatal outcome, microbial invasion of the amniotic cavity (MIAC), and fetal injury. A variety of fetal and maternal cells within the placental membranes are capable of pathogen recognition for initiating and sustaining an inflammatory response; these include amniotic epithelial cells, fetal macrophages, decidual macrophages, decidual NK cells, and neutrophils (Singh et al., 2005; Duriez, et al., 2014; Boldenow et al., 2015; Whidbey et al., 2015; Boldenow et al., 2016). While a severe infection leading to early preterm birth is typically associated with MIAC, an inflammatory response confined to the placenta even in the absence of MIAC is also sufficient to induce preterm labor in some cases (Adams Waldorf et al., 2011). Interestingly, intra-amniotic administration of cytokines, such as tumor necrosis factor-alpha (TNF-α) and inter-leukin (IL)-1b alone (i.e., without any bacteria), can induce preterm labor in pregnant nonhuman primates (Sadowsky et al., 2006), and IL-1a, IL-1b, IL-6, and IL-8 drive infection-associated preterm birth in humans (reviewed by Cappelletti et al., 2016). Thus, placental inflammation induced by bacterial infection is likely a critical component of infection-associated preterm birth. Also, bacterial suppression of placental immune responses could contribute to MIAC, leading to stillbirths. A better under-standing of the mechanisms by which in utero GBS infections drive preterm births or stillbirths may lead to the development of new interventions to reduce the burden of disease. Key bacterial and host factors that have been identified to influence GBS colonization and perinatal infection are described in the next section.

II.2.6. Bacterial factors that promote GBS vaginal colonization, ascending infection, and preterm birth

II.2.6.1. GBS adhesins to extracellular matrix (ECM) proteins

As an opportunistic commensal constituting a part of the intestinal and vaginal physiologic microbiota, GBS colonization, persistence, translocation, and invasion of host barriers are largely dependent on their adherence abilities to host cells and ECM (Singh et al., 2012; Landwehr-Kenzel and Henneke, 2014). Functionally characterized adhesins mediating GBS adherence and/or invasion within the host are the fibrinogen-binding proteins (Fbs), the laminin-binding protein (Lmb), the group B streptococcal C5a peptidase (ScpB), the streptococcal fibronectin-binding protein A (SfbA), and the

GBS immunogenic bacterial adhesin (BibA). In addition, surface-protruding structures comprised of multiples genes like pili are considered as essential adhesins in promoting GBS colonization, persistence, biofilm production, and central nervous system invasion. Major adhesins mediating GBS interaction with host cells are depicted in Figure 3.

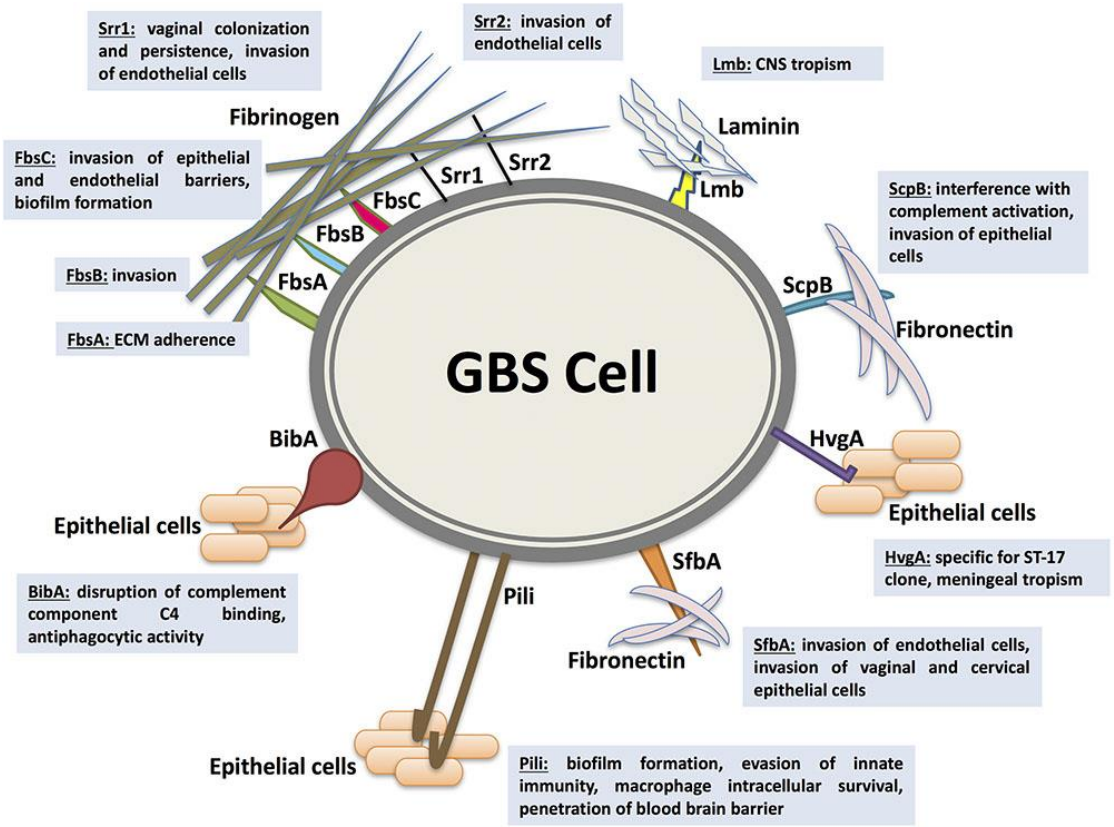


Figure 3. Major adhesins mediating *S. agalactiae* (GBS) interaction with host cells. Source: Shabayek and Spellerberg (2018).

Up to date, five Fbs have been characterized in GBS; FbsA (Schubert et al., 2004), FbsB (Gutekunst et al., 2004), the serine-rich repeat glycoproteins Srr1 and Srr2 (Seo et al., 2012, 2013), and recently FbsC or BsaB (Buscetta et al., 2014; Jiang and Wessels, 2014). In general, invasive GBS isolates display stronger fibrinogen-binding abilities in comparison to colonizing ones (Rosenau et al., 2007). FbsA was mainly shown to promote adherence (Schubert et al., 2004) whereas FbsB was shown to be required for invading human cells (Gutekunst et al., 2004). Srr1 and Srr2 were reported to mediate invasion of microvascular endothelial cells (Seo et al., 2012, 2013). Additionally, Srr1 was demonstrated to promote vaginal colonization and persistence, since a Srr1-deficient mutant displayed reduced persistence in a mouse GBS vaginal colonization model (Sheen et al., 2011). FbsC was recently characterized to promote invasion of epithelial and endothelial barriers. FbsC deletion mutant of GBS displayed a drastic reduction in abilities for adherence, invasion and biofilm formation. Besides, virulence abilities of FbsC deletion mutant were impaired in murine infection models (Buscetta et al., 2014). Interestingly, the fibrinogen-binding abilities of the hypervirulent CC17

clones are mainly attributable to FbsB more than FbsA. Deletion mutants of *fbsB* displayed 78–80% reduction in their binding abilities vs. 49–57% as encountered with *fbsA* deletion mutants of CC17 strains (Al Safadi et al., 2011). Accordingly, the relative transcription level of *fbsB* was up to 12.7-fold higher than *fbsA* gene in CC17 stains (Al Safadi et al., 2011). Moreover, Srr2 was highly expressed and exclusively detected in ST-17, however, Srr1 was absent (Seifert et al., 2006; Seo et al., 2013). Furthermore, CC17 strains are devoid of FbsC. The *fbsC* gene is not adequately expressed in CC17 strains because of a lineage-dependent frameshift mutation (Buscetta et al., 2014).

In addition to the Fbs family, the Lmb adhesin appears to have a pronounced role in bacterial tropism of the central nervous system. Spellerberg et al. (1999) reported Lmb to be essential for GBS colonization of damaged epithelium and subsequent translocation into the bloodstream. This role was later confirmed by Tenenbaum et al. (2007) as they demonstrated mutation of the *lmb* gene to result in a dramatic reduction in GBS invasion of the brain microvascular endothelial cells. In consistence, Al Safadi et al. (2010) displayed higher expression levels of Lmb in GBS strains associated with meningitis in comparison to other isolates whereas the expression levels of other ECM-binding proteins, such as ScpB mediating fibronectin binding ability, remained unchanged.

ScpB or the group B ScpB is a surface associated serine protease that both interrupts complement activation through splitting the neutrophil chemoattractant C5a and mediates bacterial binding to fibronectin (Chmouryguina et al., 1996; Bohnsack et al., 1997; Cheng et al., 2002; Lindahl et al., 2005). The fibronectin binding ability conferred by the *scpB* gene appears to be involved in cellular adherence and invasion. In frame deletion mutation of *scpB* gene significantly reduced invasion of human epithelial cells *in vitro* (Cheng et al., 2002). Strikingly, *scpB* and *lmb* genes were found to be encoded on a composite transposon where the *scpB* gene is positioned directly upstream of the *lmb* gene. The *scpB-lmb* intergenic region has been described as a hot spot for integration of the GBS mobile genetic elements GBSil and IS*1548* which are located in the promoter region of the *lmb* gene (Franken et al., 2001; Granlund et al., 2001; Luan et al., 2003, 2005; Broker and Spellerberg, 2004). Al Safadi et al. (2010) reported a marked increase in the transcription levels of *lmb* gene for invasive GBS isolates carrying IS*1548* in the *scpB-lmb* intergenic region associated with an increased laminin binding ability. However, no influence was observed on the *scpB* gene. Deletion mutation of IS*1548* revealed IS*1548* to act as an *lmb* gene up-regulator when compared to the wild-type parent strains. Interestingly the ability of GBS to colonize human mucosal surfaces seems to be closely linked to the presence of this composite transposon carrying *scpB* and *lmb*. In a large percentage of bovine strains, the encoded genes are absent while the presence in human colonizing strains, as well as invasive strains, is close to 100% (Franken et al., 2001; Sørensen et al., 2010; Rato et al., 2013).

More recently, a novel GBS fibronectin binding protein has been identified (Mu et al., 2014). It was designated as streptococcal fibronectin-binding protein A (SfbA) and reported to be highly conserved in GBS mediating cellular invasion but not adherence. SfbA was shown to be directly involved in fibronectin binding and human brain microvascular endothelial cells invasion. When expressed in recombinant non-pathogenic *Lactococcus lactis*, fibronectin binding ability was significantly greater in comparison to a SfbA negative control strain. The investigation also demonstrated SfbA to be primarily involved in brain microvascular endothelial cells invasion. Infection of mice with *sfbA* mutants resulted in a reduced ability to breach the blood brain barrier and subsequent meningitis. This is supported by a study showing SfbA to be crucial for invasion of astrocytes which are physically associated with the brain endothelial cells (Stoner et al., 2015). Furthermore, SfbA contributes to GBS invasion of vaginal and cervical epithelial cells and hence may take part in GBS colonization and niche establishment in the vagina (Mu et al., 2014). Another fibronectin binding protein was described in 2014 (Jiang and Wessels, 2014), BsaB or the bacterial surface adhesin of GBS is a fibronectin and laminin-binding protein which is involved in GBS binding to epithelial cells and in biofilm formation. Deletion of *bsaB* gene and a cotranscribed upstream region significantly abrogated GBS adherence to VK2 vaginal epithelial cells *in vitro* and immobilized fibronectin. However, genome and sequence analysis revealed BsaB and FbsC as identical proteins encoded by the same gene (Buscetta et al., 2014). The obtained results are in agreement with those of Jiang and Wessels (2014), except that FbsC or BsaB was found to mediate GBS attachment to fibrinogen instead of fibronectin. Hence, BsaB was renamed to FbsC.

The multitude of GBS adhesins allowing attachment to different ECM, stresses the importance of this step in GBS pathogenesis, which was confirmed in different *in vivo* models. In this regard, fibrinogen binding may play an especially important role as demonstrated by the presence of numerous fibrinogen binding proteins. These may represent a kind of "backup" system in cases where the primary fibrinogen adhesin was rendered non-functional.

II.2.6.2. GBS adhesins to cellular targets

Besides adherence to ECM, the adhesion to host cells plays an important role in the pathogenesis of GBS. An essential adhesin in this context is the GBS immunogenic bacterial adhesin (BibA). It is a cell wall-anchored protein which is well-conserved in GBS and is involved in bacterial binding to human epithelial cells (Santi et al., 2007, 2009). A knockout mutant displayed impaired adherence capacity to the lung, intestinal, and cervical epithelial cells (Santi et al., 2007). Overexpression of BibA resulted in increased adherence to human epithelial cells in recombinant wild-type strains harboring a *bibA* plasmid (Santi et al., 2007). In addition, BibA was reported to aid GBS survival in human blood through interfering with the classic complement pathway by binding the C4-binding protein and by conferring anti-phagocytic activity against opsonophagocytic

killing by human neutrophils (Santi et al., 2007, 2009). A total of four variants of BibA (I, II, III, and IV) were described in GBS (Brochet et al., 2006; Santi et al., 2007, 2009). Interestingly, variant IV, which was found to be highly similar to the bovine BibA counterparts, was exclusively associated with ST-17 strains (Lamy et al., 2006; Santi et al., 2009). Thus, BibA seems to be a multifactorial virulence factor in regard to GBS as a pathobiont. It contributes to GBS mucosal colonization and adherence to host cells and then confers resistance to phagocytic killing at a stage when the switch to invasive GBS infection has occurred.

The GBS hypervirulent adhesin (HvgA) is a novel cell wall anchored protein that is specific for the hypervirulent clone ST-17. It was first described (Tazi et al., 2010) as being strongly associated with ST-17 causing neonatal meningitis in LO disease. It was suggested to promote meningeal tropism in neonates through efficient intestinal colonization and subsequent translocation across the intestinal and the blood brain barriers. Bypassing intestinal colonization by intravenous infection resulted in a significant decrease in the amount of bacteria reaching the central nervous system. HvgA was required for intestinal colonization in orally infected mice for meningitis development. In addition, HvgA was found to mediate GBS adherence to intestinal epithelial cells, choroid epithelial cells and microvascular endothelial cells (Tazi et al., 2010). Clones expressing HvgA exhibited greater adherence abilities than non-expressing ones. HvgA thus contributes to colonization as well as invasion of hypervirulent clones (Tazi et al., 2010).

II.2.6.3. GBS pili

Pili are also crucial for GBS adhesion. Different from their Gram-negative counterparts, pili in GBS have been shown to be primarily involved in epithelial cell colonization, biofilm formation, translocation, and invasion. Pili are cell-wall anchored appendages extending from the bacterial surface. They contain covalently linked multimeric motifs that are composed of three pilin proteins, the pilus shaft backbone protein (BP) or PilB subunits, and the two ancillary proteins AP1, AP2 located at the pilus tip (PilA subunit, the pilus-associated adhesin) and pilus base (PilC subunit, the pilus anchor), respectively, (Dramsi et al., 2006; Rosini et al., 2006; Maisey et al., 2007, 2008; Cozzi et al., 2015). While PilB has been shown to be involved in bacterial invasion and paracellular translocation mediating resistance to phagocytic killing and virulence, PilA was found contributing to cellular adherence and colonization (Dramsi et al., 2006; Krishnan et al., 2007; Maisey et al., 2007, 2008; Pezzicoli et al., 2008; Sheen et al., 2011). Three pilus variants named PI-1, PI-2a, PI-2b were reported in GBS representing two pilus islands (PI) where PI-2a and PI-2b are variants of the pilus island 2 (PI-2). All characterized GBS strains harbored at least one variant or a combination of two pilus islands (Rosini et al., 2006; Margarit et al., 2009; Springman et al., 2014). PI-1 pili were also found to play an important role in evasion of innate immunity mechanism. They diminished macrophage-mediated phagocytic killing of GBS by 50%

with no influence on complement-promoted opsonophagocytic killing by neutrophils (Jiang et al., 2012). Strikingly, PI-1 pili do not appear to contribute to bacterial adhesion to lung, vaginal or cervical epithelial cells (Jiang et al., 2012). The PI-2a pili were found to have a specific involvement in adherence and biofilm formation and not PI or PI-2b (Konto-Ghiorghi et al., 2009; Rinaudo et al., 2010). The PI-2b protein, however, was demonstrated to increase the intracellular survival in macrophage (Chattopadhyay et al., 2011). In addition, a special role for pilus type 2b has been suggested in promoting strain invasiveness and bacterial host cell interactions. Mutants of pilus 2b possess less adherence and invasion capacities for epithelial and endothelial cells (Lazzarin et al., 2017). Pilus 2b was further identified as important for infection and penetration of the blood brain barrier. These results are supported by an investigation of the distribution of pilus islands among GBS strains belonging to ST lineages of human and bovine origin (Springman et al., 2014). In addition, the distribution of pili islands appears to determine the capacity for colonization or invasive infections. Invasive GBS were more likely to carry a combination of PI and one of the PI-2 variants in comparison to maternal colonizing isolates. Moreover, GBS causing invasive neonatal disease including all CC-17 strains were harboring PI-1 plus PI-2b. Earlier genomic studies showed pilus type 2b to be conserved in the ST-17 hypervirulent clone (Brochet et al., 2006). Interestingly PI-2b pilus variants are almost exclusively present in bovine GBS isolates. These bovine strains mostly lack PI-1, unlike the human isolates which commonly encode the pilus PI-1 in association with one of the PI-2 variants (Springman et al., 2014).

II.2.6.4. Biofilm formation

Colonization and persistence in different host niches is dependent on the adherence capacity of GBS to host cells and tissues. This then facilitates bacterial cell aggregation and formation of sessile communities known as biofilms. Bacterial biofilms represent well-known virulence factors with a vital role in persistence and chronic infections. In the host environment, bacteria are often protected from the immune system by building sessile colonies embedded in an extracellular matrix of polysaccharides representing the biofilm. For GBS the bacterial capsule and type IIa pili have been demonstrated to play an important role in biofilm formation (Konto-Ghiorghi et al., 2009; Xia et al., 2015). Host environmental conditions are crucial determinants in developing bacterial biofilms (Costerton et al., 1999; Lewis, 2005; Nobbs et al., 2009; Rosini and Margarit, 2015). Contradictory data are available concerning the environmental cues favoring biofilm communities in GBS (Rosini and Margarit, 2015). As a normal inhabitant of the vagina, acidic pH seems to be optimal for GBS colonization. Early investigations reported enhanced GBS adherence to vaginal epithelial cells under low pH in comparison to neutral pH (Zawaneh et al., 1979; Tamura et al., 1994). In line with these observations, a significantly higher biofilm production of colonizing GBS isolates from pregnant women was demonstrated at pH 4.5 vs. pH 7 (Ho et al., 2013). Similarly, enhanced biofilm formation of GBS was shown under acidic pH conditions in comparison to neutral pH with the strongest biofilm producing GBS

isolates belonged to the ST-17 sequence type. In respect to GBS origins, higher frequencies of strong biofilm producers were found among neonatal strains in comparison to colonizing strains (D'Urzo et al., 2014). However, a recent investigation reported invasive GBS belonging to CC17 and CC19 lineages as weak biofilm formers while GBS isolated from asymptomatic carriers were found to be strong biofilm producers (Parker et al., 2016). One possible explanation for this discrepancy is the experimental set up of the study since GBS biofilm formation was tested at neutral pH conditions and not under acidic pH. Furthermore, the presence of human plasma was shown to promote GBS biofilm formation (Xia et al., 2015).

In summary, biofilms allow long-term bacterial persistence and protect bacteria from recognition by the immune system. For GBS low pH and the presence of plasma appear as crucial environmental factors through controlling the expression of bacterial surface-associated structures, such as pili and the capsule, which are both involved in promoting bacterial biofilm formation.

II.2.6.5. Hemolytic pigment

GBS is a *b*-hemolytic bacterium, and the hemolytic property of GBS is important for infection and immune evasion. Hemolytic activity of GBS is due to the ornithine rhamnolipid pigment (hereafter referred to as 'hemolytic pigment' or 'pigment') (Whidbey et al., 2013), which is produced by the genes of the *cyl* operon (Pritzlaff et al., 2001). Transcription of *cyl* genes, and therefore production of the hemolytic pigment, is negatively regulated by the CovR/S two-component system (also known as CsrR/ S) (Lamy et al., 2004; Jiang et al., 2005; Whidbey et al., 2013). Consequently, deletion of covR/S renders GBS hyperhemolytic and hyperpigmented (Lamy et al., 2004; Jiang et al., 2005; Whidbey et al., 2013). Conversely, deletion of the *cylE* gene, which encodes an N-acyltransferase necessary for pigment production (Whidbey et al., 2013), renders GBS nonpigmented and nonhemolytic (Pritzlaff et al., 2001; Whidbey et al., 2013). Identification of hemolytic, hyperhemolytic and nonhemolytic GBS strains in human cases allowed for a greater understanding of the role of hemolysin in GBS infection.

The hemolytic pigment promotes GBS penetration of human placenta (chorioamniotic membranes) and induces loss of barrier function in human amniotic epithelial cells (Whidbey et al., 2013). Furthermore, hyperpigmented GBS strains were isolated from either the amniotic fluid or chorioamniotic membranes of women in preterm labor (Whidbey et al., 2013). Randis et al. (2014) also noted decreased bacterial dissemination, fetal injury, and preterm birth in mice that were vaginally inoculated with non-hemolytic GBS (i.e., GBS lacking *cylE*). Recently, it has been shown that the increased hemolytic pigment expression accelerated GBS invasion of the amniotic cavity with significant uterine contractions and inflammatory responses indicative of preterm labor in a nonhuman primate model (Boldenow et al., 2016). Although infection with hyperpigmented GBS induced the formation of neutrophil extracellular traps

(NETs) in chorioamniotic membranes of nonhuman primates (Boldenow et al., 2016), these GBS strains were resistant to the antimicrobial activity of NETs, likely through increased pigment-mediated antioxidant activity (Liu et al., 2004). Formation of NETs in response to GBS infection was also observed in murine models of colonization (Carey et al., 2014) and ascending infection (Kothary et al., 2017). Collectively, these studies indicate a role for the hemolytic pigment in promoting GBS dissemination in uterine, placental, and fetal tissues during pregnancy.

The GBS hemolytic pigment also affects vaginal colonization. Absence of the hemolytic pigment reduced the ability of GBS to successfully colonize the vagina, possibly due to increased susceptibility to neutrophil clearance (Randis et al., 2014; Carey et al., 2014). Surprisingly, hyperpigmented GBS also exhibited decreased vaginal colonization in mice (Patras et al., 2013; Gendrin et al., 2015; Patras and Doran, 2016), likely due to increased pigment-mediated stimulation of neutrophil (Patras et al., 2013) and mast cell (Gendrin et al., 2015) inflammatory pathways. Consistent with these observations, hyperpigmented strains of GBS were rarely isolated from rectovaginal swabs of asymptomatic pregnant women (Gendrin et al., 2015). These results emphasize the role of vaginal immune responses in pathogen colonization.

Apart from immune cells, pH regulates GBS gene expression and therefore influences vaginal colonization. For instance, the GBS CovR/S system responds to pH wherein increased CovR/S regulation was observed under low (acidic) pH (Santi et al., 2009; Cumley et al., 2012; Park et al., 2012). Changes in pH also influence GBS adhesion (Tamura et al., 1994; Park et al., 2012), survival (Borges et al., 2012), and biofilm formation (Ho et al., 2013; D'Urzo et al., 2014). Of note, high vaginal pH and a non-lactobacilli-dominated vaginal microbiome (Ravel et al., 2011) were associated with a higher incidence of GBS vaginal colonization (Hickman et al., 1999; Kwatra et al., 2016) and neonatal disease (Lin et al., 2003; Weston et al., 2011; Peltier et al., 2012) in women of African descent. These studies indicate that GBS responds to environmental cues, such as pH, and even utilizes regulatory systems such as CovR/S to temporally control virulence factor expression (e.g., hemolytic pigment) during pregnancy-associated infections. As such, this makes the GBS pigment an intriguing target for vaccine development.

II.2.6.6. Hyaluronidase

The GBS hyaluronidase, known as HylB, promotes vaginal colonization (Kolar et al., 2015). HylB is secreted by GBS and specifically targets and degrades host hyaluronic acid (Gochnauer and Wilson, 1951; Baker and Pritchard, 2000). Hyaluronic acid is an extracellular matrix glycosaminoglycan composed of repeating disaccharide units (N-acetyl- D-glucosamine-D-glucuronic acid) and is important for cell migration, cell signaling, regulation of inflammation, and the prevention of ascending infection (Mahendroo, 2012; Akgul et al., 2014). GBS HylB degrades host hyaluronic acid into

its disaccharide components, which are immunosuppressive as they bind to TLR2/TLR4 receptors and block signaling (Kolar et al., 2015). Deletion of HylB led to increased clearance of GBS from the mouse vagina (Kolar et al., 2015). Similarly, GBS lacking HylB was less able to ascend from the vagina to the uterus and was diminished for its ability to invade fetal tissues and cause preterm birth (Vornhagen et al., 2016). By contrast, uterine tissue infected with HylB-proficient GBS showed decreased levels of inflammatory cytokines such as TNF-a, IL-6, and IL-8, leading to bacterial ascension (Vornhagen et al., 2016). Thus, suppression of key inflammatory responses also plays an important role in GBS infection-associated fetal injury.

II.2.6.7. Other virulence factors

Recent studies have described a role for extracellular membrane vesicles (MVs) in the weakening of placental membranes (Surve et al., 2016). GBS MVs contained multiple virulence factors, including: (i) HylB; (ii) CAMP factor (Christine, Atkins, Munch-Peterson factor; Christie et al., 1944), a secreted pore-forming protein (Lang and Palmer, 2003) that may amplify (Jurgens et al., 1987), but is not essential for, GBS virulence (Hensler et al., 2008); (iii) IgA-binding protein, with the ability to bind human IgA (Kvam et al., 1992) for host immune evasion (Nordstrom et al., 2011), and (iv) multiple enzymes that may regulate ECM degradation (Surve et al., 2016). Intra-amniotic administration of GBS MVs in pregnant mice caused significant damage to choriodecidual tissues and stimulated leukocytic infiltration and inflammation, leading to membrane weakening (Surve et al., 2016). The specific role played by each virulence factor in the context of MVs remains unknown. MV weakening of choriodecidual membranes represents a novel mechanism of GBS fetal injury.

II.2.7. Host determinants of GBS vaginal colonization, ascending infection, and preterm birth

II.2.7.1. Vaginal colonization

Evasion of the host immune response is essential for successful vaginal colonization. GBS vaginal immunity is mediated by its ability to resist many physical and cellular barriers, including the luminal mucus layer, vaginal epithelia, and immune cells in the vagina. Recent work, using animal models, has provided new information regarding the host immune response to GBS vaginal colonization (Patras et al., 2013; Carey et al., 2014; Patras et al., 2015; Gendrin et al., 2015). Vaginal immune responses to GBS are largely mediated by neutrophils (Patras et al., 2013; Carey et al., 2014; Patras et al., 2015), mast cells (Gendrin, C. et al., 2015), and macrophages (Carey et al., 2014) (Figure 4). The role of NK cells and dendritic cells in GBS colonization is not known. Multiple soluble inflammatory cytokines and chemokines have been identified as important for reducing GBS vaginal colonization and include IL-1b, IL-6, IL-8, IL-17, IL-23, and histamine (Patras et al., 2013; Carey et al., 2014; Patras et al., 2015). Currently, the mucosal T cell response to GBS colonization is ill-defined. Studies have

shown that IL-17 and IL-17+ cells play an important role in clearance of a hyperadherent and invasive GBS strain from the vagina (Patras et al., 2015), suggesting that the Th17 differentiation pathway is important for controlling persistent GBS colonization. Similarly, another study found cytokines involved in Th1, Th2, and Th17 differentiation pathways as important for decreased colonization (Carey et al., 2014); however, T cells were not directly identified as being important in either study.

II.2.7.2. Infection of placental membranes

Many studies have focused on GBS infection of the placental membranes (chorioamnion) in order to understand how GBS penetrates these barriers and induces chorioamnionitis. GBS is able to adhere to and invade both chorionic and amniotic epithelial cells (Winram et al., 1998). Adherence and invasion to these cells are mediated by several factors: (i) IagA, a glycosyltransferase that helps anchor lipoteichoic acid to the cell surface (Doran et al., 2005); (ii) the hemolytic pigment and its regulator CovR/S (Whidbey et al., 2013); and (iii) quorum sensing mediated by genes in the rgf operon (Parker et al., 2017). GBS has also been shown to induce secretion of multiple cytokines and defensins from placental membranes *ex vivo*, including TNF-a, IL-1a, IL-1b, IL-6, and IL-8 (Flores-Herrera et al., 2012; Boldenow et al., 2013; Boldenow et al., 2015). Inflammation is stimulated either through pattern-recognition receptor (PRR) sensing of GBS antigens (Boldenow et al., 2015) or by pigment-mediated activation of nuclear factor-kβ (NF-kβ) (Whidbey et al., 2013). An important family of PRRs that mediate placental membrane immunity are the Siglecs (Brinkman-Van der Linden et al., 2007¸ Ali et al., 2014), a family of cell-surface sialic acid-binding lectins that regulate innate and adaptive immune function (Crocker et al., 2007). GBS is able to bind Siglecs through the sialic acid capsule or b-protein to suppress immune cell activation (Carlin et al., 2007; Carlin et al., 2009; Ali et al., 2014) and placental membrane inflammation (Ali et al., 2014), potentially leading to increased rates of GBS-associated preterm birth and stillbirth.

II.2.7.3. Fetal injury

GBS invasion of the fetus *in utero* leads to a variety of adverse outcomes, including tissue damage, inflammation, lung and brain injury, pneumonia, meningitis, sepsis, and fetal death. GBS can invade multiple fetal organs, including the lung, blood, liver, spleen, and gastrovascular cavity. Fetal tissue damage has been observed in the presence and absence of bacterial invasion, which may be due to inflammation in the gestational tissues and amniotic fluid. GBS invasion of fetal tissues induces inflammation and fetal death (Monari et al., 2013; Randis et al., 2014; Whidbey et al., 2015; Vornhagen et al., 2016), and in the case of the hemolytic pigment, this involved induction of the NLRP3 inflammasome (Whidbey et al., 2015).

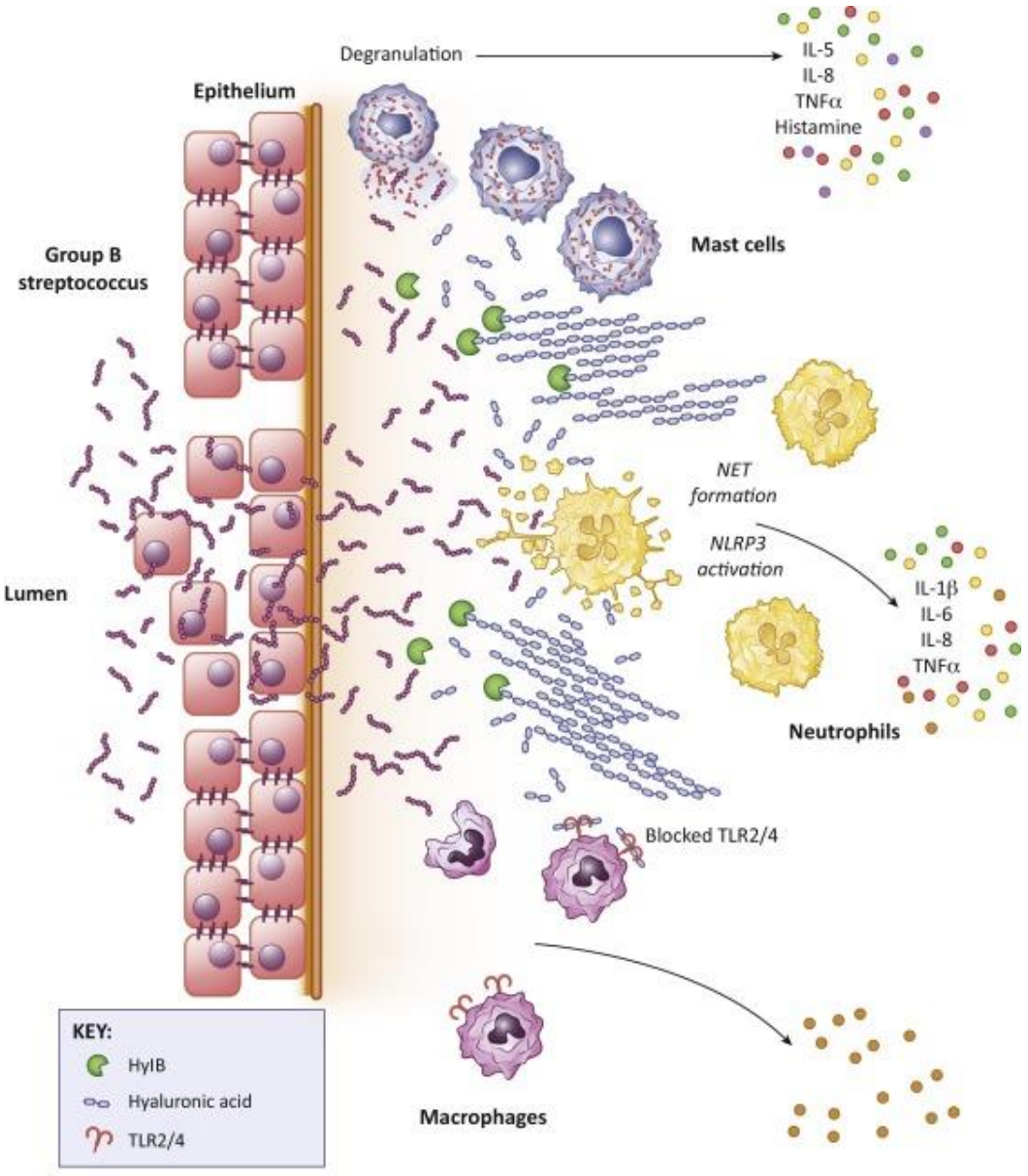


Figure 4. Interaction of GBS with innate immune cells during genital infection. Source: Vornhagen et al. (2017).

GBS stimulates the NLRP3 inflammasome in a number of immune cells, such as dendritic cells (Costa et al., 2012), macrophages (Whidbey et al., 2015), and neutrophils (Mohammadi et al., 2016), which contribute to *in vivo* inflammation. Interestingly, fetal injury is not entirely dependent on bacterial invasion of fetal tissues. Fetal lung injury can also be caused by GBS-induced chorioamnionitis without bacterial invasion (Adams Waldorf et al., 2011). Also, increases in amniotic fluid cytokines contribute to fetal lung injury (Adams Waldorf et al., 2011) and dysregulation of fetal lung development (Adams Waldorf et al., 2015). Moreover, intra-amniotic administration of MVs leads to significantly increased rates of fetal damage and preterm birth (Surve et al., 2016). These studies suggest that fetal injury can occur during transient or limited infection, and

further emphasizes the importance of developing therapeutics that prevent vaginal colonization and ascending infection.

II.2.8. Preventive measures: intrapartum antibiotic prophylaxis (IAP)

The relevance of GBS as agent of neonatal infections prompted the finding of strategies for its eradication from the intestinal and genitourinary mucosal surfaces of pregnant women (Puopolo et al., 2005). Chlorhexidine vaginal treatment, with or without neonatal wash, reduced GBS bacterial load but showed no impact on EOD (Stade et al., 2004). Induction of labor with intravenous oxytocin may be preferable for GBS positive women with prelabor rupture of membranes at term as infections are reduced. However, the most commonly used prevention intervention is intrapartum chemoprophylaxis with antibiotics to mothers with known GBS colonization. To date, four approaches have been recommended for the prevention of neonatal GBS infections: (a) a risk-based strategy; (b) a screening- (vaginal/rectal GBS cultures) based strategy; (c) a combined risk/screening-based strategy; and (d) a combined risk/screening-based strategy using the PCR test (Akker van Marle, 2005).

In 1976, chemoprophylaxis was first proposed for reducing maternal GBS colonization in labor to reduce neonatal disease (Ablow et al., 1976). Non-randomized studies showed that intravenous ampicillin given during labor to GBS positive women could significantly reduce neonatal GBS colonization, and a non-significant reduction in GBS neonatal invasive disease was reported (Yow et al., 1979; Allardice et al., 1982). In 1992, the first guidelines for GBS prevention were published in the US (AAP, 1992; ACOG, 1992). Since then numerous guidelines with different recommendations have been published by various organizations (AAP, 1997; ACOG, 1996; CDC, 1996; SOGC, 1997; CDC, 2002; RCOG, 2003; SOGC, 2004; CDC, 2010; Alós Cortés, et al., 2013; Money, 2013), often based on studies of poor quality. Some authors and organisms have claimed a temporary association between the introduction of GBS screening guidelines and a decline in the GBS EO disease rate (CDC, 2005; CDC, 2007). The incidence of invasive EO GBS disease decreased from 1.8 cases/1,000 live births in the early 1990s to 0.3 cases/1,000 live births in 2010 (Andreu et al., 2003). In contrast, no reduction in rates of LO GBS disease has been observed since then (CDC, 2007). Currently in the USA and other countries with a GBS screening-based strategy, penicillin is the drug of choice for intrapartum prophylaxis given every 4 h intravenously until the baby is born. Information on whether intrapartum ampicillin is preferable to penicillin for GBS colonized women is lacking (Ohlsson and Shah, 2013).

However, the same literature has been interpreted differently by different scientists and professional organizations and this have relevant implications for clinical practice. In the three studies investigating the effects of intrapartum antibiotics versus no treatment for GBS-colonized women, risks of bias for one or more key domains in the study methodology and execution were identified and such risks were high enough to

affect the interpretation of the results (Ohlsson and Shah, 2009; Ohlsson and Shah, 2013; Ohlsson and Shah, 2014). In fact, these reviews based on Cochrane's guidelines have concluded that there is no valid information from these biased trials to inform clinical practice.

The conclusion of Cochrane's reviews on this issue is that intrapartum chemoprophylaxis to reduce perinatal GBS infections are not supported by conclusive evidence from well designed and conducted randomised controlled trials (Ohlsson and Shah, 2009):

"*Women, men and children of all ages can be colonized with Group B streptococcus (GBS) bacteria without having any symptoms; About one in 2000 newborn babies have Group B streptococcus bacterial infections, usually evident as respiratory disease, general sepsis, or meningitis within the first week. The baby contracts the infection from the mother during labor. Giving the mother an antibiotic directly into a vein during labor causes bacterial counts to fall rapidly, which suggests possible benefits but pregnant women need to be screened. Many countries have guidelines on screening for GBS in pregnancy and treatment with antibiotics. However, very few of the women in labor who are GBS positive give birth to babies who are infected with GBS and antibiotics can have harmful effects such as severe maternal allergic reactions, increase in drug-resistant organisms and exposure of newborn infants to resistant bacteria, and postnatal maternal and neonatal yeast infections.*

This review finds that giving antibiotics is not supported by conclusive evidence. The review identified four trials involving 852 GBS positive women. Three trials, which were around 20 years old, compared ampicillin or penicillin to no treatment and found no clear differences in newborn deaths although the occurrence of early GBS infection in the newborn was reduced with antibiotics. Maternal colonization with group B streptococcus (GBS) during pregnancy increases the risk of neonatal infection by vertical transmission. Administration of intrapartum antibiotic prophylaxis (IAP) during labor has been associated with a reduction in early onset GBS disease (EOGBSD). However, treating all colonized women during labor exposes a large number of women and infants to possible adverse effects without benefit. All cases of perinatal GBS infections are unlikely to be prevented even if an effective vaccine is developed.

Intrapartum antibiotic prophylaxis appeared to reduce EOGBSD, but this result may well be a result of bias as we found a high risk of bias for one or more key domains in the study methodology and execution. There is lack of evidence from well designed and conducted trials to recommend IAP to reduce neonatal EOGBSD. Ideally the effectiveness of IAP to reduce neonatal GBS infections should be studied in adequately sized double-blind controlled trials. The opportunity to conduct such trials has likely

been lost, as practice guidelines (albeit without good evidence) have been introduced in many jurisdictions"

It should be noted that the guidelines have changed many times, indicating that they are not based on clear evidence informing best clinical practice (Ohlsson and Shah, 2014).

Possibly, the strongest argument supporting that introduction of GBS screening and IAP is not associated to the reduction of EOS by GBS is the fact that the rate has declined similarly, at least, in some European countries in which GBS screening strategy was not adopted. As an example, the UK National Screening Committee examined the issue of strategies for the prevention of EOS GBS disease in November 2008 and recommended that routine screening using bacteriological culture or near-patient testing techniques should not be introduced into UK practice (UK National Screening Comité, 2012). The latest update of the guidelines from the Royal College of Obstetricians and Gynaecologist regarding GBS do not recommend routine bacteriological screening of all pregnant women for antenatal GBS carriage (RCOG, 2012). Previously, an analysis of the cases of GBS sepsis occurred in UK and Ireland among the ~800,000 neonates born in a year (with ~198.000 GBS-colonized women) revealed that 840 GBS-positive women should be treated to avoid a single neonatal GBS sepsis (Heath et al., 2004). This fact was considered unacceptable in the frame of the current antibiotic resistance epidemics.

II.2.9. Adverse effects of IAP

The first critical review of randomized controlled trials of intrapartum chemoprophylaxis of perinatal GBS infections identified numerous methodological flaws (Ohlsson, 1992). In addition, such strategy for GBS management in pregnancy has been questioned (Yudin et al., 2006). Therefore, a Cochrane review adopting high-quality methodology was justified. In the first Cochrane review on the efficacy of IAP to eradicate GBS colonization, Ohlsson and Shah (2009) considered that it was important to know if intrapartum antibiotics do more good than harm in trying to reduce mortality and morbidity from neonatal GBS infections. As stated above, most women colonized with GBS are asymptomatic, so screening is necessary if these women are to be identified. However, of the women in labor who are GBS positive, very few will give birth to babies who are infected with GBS. Hence, giving intravenous antibiotics to all women in labor who are GBS positive will put a large number of women and babies at risk of adverse effects unnecessarily. These adverse effects include potentially fatal anaphylaxis, increase in drug-resistant organisms and the medicalization of labor and the neonatal period (RCOG, 2003). Severe allergic reaction to antibiotics has been reported among mothers giving birth (Berthier et al., 2007; Jao et al., 2006).

However, the biggest concerns are the increasing rates of antibiotic resistance among clinically relevant microorganisms and the impact of IAP on the acquisition,

composition and development of the infant microbiota. In the words of Blaser (2016): "*Anti-infectives, including antibiotics, are essentially different from all other drugs; they not only affect the individual to whom they are given but also the entire community, through selection for resistance to their own action. Thus, their use resides at the intersection of personal and public health. Antibiotics can be likened to a four-edged sword against bacteria. The first two edges of the antibiotic sword were identified immediately after their discovery and deployment in that they not only benefit an individual in treating their infection but also benefit the community in preventing the spread of that infectious agent. The third edge was already recognized by Alexander Fleming in 1945 in his Nobel acceptance speech, which warned about the cost to the community of antibiotic resistance that would inevitably evolve and be selected for during clinical practice. We have seen this cost mount up, as resistance curtails or precludes the activities of some of our most effective drugs for clinically important infections. But the fourth edge of the antibiotic sword remained unappreciated until recently, i.e., the cost that an antibiotic exerts on an individual's own health via the collateral damage of the drug on bacteria that normally live on or in healthy humans: our microbiota. These organisms, their genes, metabolites, and interactions with one another, as well as with their host collectively, represent our microbiome. Our relationship with these symbiotic bacteria is especially important during the early years of life, when the adult microbiome has not yet formed*".

The incidence of postnatal maternal and neonatal bacterial and yeast infections may increase with the use of intrapartum antibiotics (Edwards et al., 2002; Dinsmoor et al., 2005; Barcaite et al., 2008). The ever evolving nature of the mechanisms conferring antibiotic resistance is particularly worrying since the selective pressure exerted by antibiotics may favour that certain bacteria (*E. coli*, *Klebsiella* spp., *Proteus* spp., *Serratia* spp., group A and group viridans streptococci, *S. aureus*, *S. epidermidis*, *Clostridium difficile*, etc.) with the potential to cause neonatal sepsis occupy the ecological niche of GBS (Schrag et al., 2006; Chung et al., 2007). Parallel, the potential spread of penicillin-resistant GBS strains is also worrying and, in fact, an increase in such a resistance has already been observed among GBS (Chu et al., 2007; Dahesh et al., 2008).

II.2.10. Adverse effects of IAP on the acquisition of the infant microbiota and potential alternatives (vaccines, probiotics)

The process of acquisition and establishment of the microbiota in the neonate is one of the most important phenomena for the later health of the individual (Renz et al., 2012; Sommer and Bäckhed, 2013). This process contribute to developmental programming of epithelial barrier function, gut homeostasis, angiogenesis, innate and host adaptive immune functions. Additionally, the microbiota have developmental effects in other organs elsewhere in the body (Claus et al., 2008; Björkholm et al., 2009) and deep consequences in systemic metabolism and neuroendocrinology (Neuman et al.,

2015). The functional development of the brain is of particular interest because it has been shown to be highly susceptible to modulation during perinatal life (Diaz Heijtz et al., 2011; Mayer et al., 2014). As an example, there seems to be an association between common neurodevelopmental disorders, such as autism and schizophrenia, and microbial pathogen infections during the perinatal period (Finegold et al., 2002; Bilbo et al., 2005; Mittal et al., 2008).

Recent animal studies have demonstrated that the early neonatal period is the most important moment for reaching the microbiota-induced host-homeostasis; if the microbiota is absent during this critical period of life the homeostatic state of the individual will not be reached, even if the microbiota is restored at a later life stage (Hansen et al., 2012; Olszak et al., 2012; Cox et al., 2014). Thus, alterations in the microbiota development during this sensitive initial period may increase the risk of disease in later life. Indeed, human studies have shown that early microbiota alterations precede the development of disease (Kalliomaki et al., 2001; Kalliomaki et al., 2008). Therefore, the first weeks of the infant life may constitute a unique window of opportunity for microbiota modulation towards the establishment of a healthy microbial profile and later health.

The microbial colonization of the intestine starts with facultative anaerobes which contribute, by lowering the intestinal redox-potential, to the later establishment of strict anaerobic microorganisms, such as *Bifidobacterium, Bacteroides* or *Clostridium* (Wopereis et al., 2014). During the first months of life there is a significant presence of *Actinobacteria* and, in many cases, *Proteobacteria* (Turroni et al., 2012; Arboleya et al., 2012; Bergström et al., 2014; Arboleya et al., 2015) which is in contrast to the dominance of *Bacteroidetes* and *Firmicutes* during adulthood (Arumugan et al., 2011; Human Microbiome Consortium, 2012). Several factors influence the early colonization and its further development in the infant, including gestational age at birth (Barrett et al., 2013; Arboleya et al., 2015; Ruiz et al., 2016), mode of delivery (Dominguez-Bello et al., 2010; Jakobsson et al., 2014), feeding strategies (Yatsunenko et al., 2012; Backhed et al., 2015; Gómez et al., 2016) and, of course, the use of perinatal antibiotics (Fouhy et al., 2012; Faa et al., 2013; Arboleya et al., 2015; Moles et al., 2015). The neonatal colonization process has been recently reviewed by Milani et al. (2017)

It has been long known that antibiotics are responsible for dysbiosis processes in the human microbiota, leading to antibiotic-associated diarrhea and gastroenteritis, urogenital, and oral infections. It is becoming evident that antibiotherapy during pregnancy, intrapartum, and lactation alters the maternal microbiota, a fact that may have negative consequences for infant health (Murk et al., 2011; Soto et al., 2014).

Perinatal antibiotic use affects the gut microbiota development during the critical first weeks of life (Arboleya et al., 2016; Cotten, 2016). The composition of the gut microbiota of neonates whose mothers received IAP has been described as aberrant in

comparison with that of non-treated neonates (Tanaka et al., 2009; Aloisio et al., 2016). The detrimental impact of perinatal antibiotics, mainly IAP, on early life microbiota may involve a lasting effect on the individual physiology (Cox et al., 2014). The use of antibiotics may influence microbiota-host crosstalk during the neonatal period, which may have profound consequences for later health (Faa et al., 2013). Actually, perinatal antibiotics exposure has been reported to increase the risk of later disease such as allergy (Droste et al., 2000; Kozyrskyj et al., 2007; Kummeling et al., 2007; Russell et al., 2012; Chu et al., 2015). An increased risk of asthma exacerbation and hospitalization, requiring inhaled corticosteroids, in children if mothers used antibiotics during pregnancy, supports a role for bacterial ecology in pre- or perinatal life for the development of asthma (Stensballe et al., 2013).

Studies of antibiotic-induced microbiome alterations and downstream effects on the developing immune system have increased our understanding of the mechanisms underlying the associations between antibiotics and adverse outcomes. The emergence of resistant microorganisms and recent evidence linking antibiotic practice variations with health outcomes has led to the initiation of antibiotic stewardship programs (Cotten, 2016).

Given that the estimated use of IAP is over 30% of total deliveries (van Dyke tal al., 2009), greater attention should be paid to its potential impact upon the gut microbiota. This impact should be considered as a factor in the decision on whether or not to administer IAP. In the frame of the current antibiotic resistance epidemics, preventive exposure of an important percentage of mothers and infants to antibiotic may be unacceptable in the future. In other words, the use of IAP for the prevention of EOS by GBS may turn out to be an interim strategy rather than a final solution (Edwards, 2008).

Availability of effective antibiotics has revolutionized public health and has been responsible for enabling countless advancements in medical care (Spellberg et al., 2008). For example, antibiotics have been critical to the development of advances in surgery and of myeloablative therapies for cancer and to the transplantation of both solid organs and hematopoietic stem cells. Effective antibiotics have also been critical for advanced medical treatment of patients with trauma and battlefield injuries, as well as myocardial infarctions, strokes, and other illnesses that require intensive care with catheters, hyperalimentation, and mechanical ventilation. Ironically, the very advances in medical care enabled by effective antibiotic therapies have, in turn, created enormous populations of increasingly immunocompromised hosts, who develop infections caused by increasingly resistant microbes that require treatment with newer, more powerful antibiotics. Meanwhile, an equally alarming decline has occurred in the research and development of new antibiotics to deal with the threat.

We are in the midst of an emerging crisis of antibiotic resistance for microbial pathogens throughout the world (Alanis, 2005). Epidemic antibiotic resistance has been described in numerous pathogens in varying contexts, including—but not limited to—a global pandemic of methicillin-resistant *S. aureus* (MRSA) infection, the global spread of drug resistance among common respiratory pathogens, including *S. pneumoniae*, *Mycobacterium tuberculosis*, and epidemic increases in multidrug-resistant (and, increasingly, truly pan-resistant) Gram-negative bacilli (Spellberg et al., 2008). Infections caused by these and other antibiotic-resistant microbes impact clinicians practicing in every field of medicine. Given their breadth of effect and significant impact on morbidity and mortality, multidrug-resistant microbes are considered a substantial threat to public health worldwide. Therefore, biological markers unambiguously associated to a risk of neonatal GBS disease are required.

In addition, it would be advisable to develop alternative strategies. Since the 1930's, important efforts have been made in order to obtain effective anti-GBS vaccines (Johri et al., 2006). Human isolates of GBS express a capsular polysaccharide (CPS) which is a major virulence factor that helps the organism evade host defense mechanisms. Clinical trials of conjugated vaccines prepared with purified CPS types Ia, Ib, II, III, IV, V, and VII have demonstrated that these preparations are safe and immunogenic (Paoletti et al., 2000). However, these preparations do not offer protection against other GBS serotypes such as VI and VIII, which are prevalent in some parts of the world. Further advances in GBS vaccine development are likely through using the newer -omics technologies (Johri et al., 2006). A GBS maternal immunization program will reduce some adverse pregnancy outcomes and intrapartum infections in the mother in addition to early- and late-onset infections in infants. The development of GBS vaccine has been reviewed by Heath (2016) and Madhi and Dangor (2017).

Even if an effective vaccine to prevent GBS infections is developed in the future, a need for alternative strategies is still likely to be present as all women will not be immunized and the vaccine may not be effective in women giving birth preterm. In this context, the isolation, selection and characterization of safe probiotic strains with the ability to antagonize and, eventually, eradicate GBS from maternal and infant mucosal surfaces is a very attractive target. This has been the objective of this PhD Thesis.

II.3. PROBIOTICS

In recent years, scientific and clinical advances in the field of probiotics have allowed the development and commercialization of some products duly contrasted. In parallel, the demand for probiotics by consumers increasingly aware of the close relationship between our microbiota and health has also increased. Unfortunately, some companies have taken advantage of this situation to apply the term "probiotic" to products that do not fit into this concept and/or whose presumed benefits lack any

scientific basis. This misuse, intentional or not, has been favored by the absence, until the beginning of the 21st century, of an international consensus on the methodology to evaluate the efficacy and safety of these products.

In 2001, a FAO/WHO joint commission of experts recognized the need to establish guidelines for the evaluation of the efficacy and safety of probiotics (FAO/WHO, 2001). The commission proposed a definition of the term probiotic that, since then, has been the most widely accepted worldwide: "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*". In 2002, another joint working group of FAO and WHO developed guidelines with the minimum requirements necessary for a product to receive the name of probiotic (FAO/WHO, 2002). The documents derived from the work of both committees and the International Scientific Association for Probiotics and Prebiotics (ISAPP) (Hill et al., 2014) are key references to know what currently understood by probiotic.

The probiotic definition is inclusive of a broad range of microbes and applications, whilst capturing the essence of probiotics (microbial, viable and beneficial to health). The definition differentiates live microbes used as processing aids or sources of useful compounds from those that are administered primarily for their health benefits. Evidence of a health benefit is required for a probiotic, at either a strain-specific or group level, depending on the nature of the benefit. Probiotics can have different means of administration, target host species (humans and animals), target populations, target sites (gut and beyond), efficacy end points and regulatory categories. Dead microbes, microbial products, microbial components do not come under the probiotic classification (Hill et al., 2014) (Figure 5).

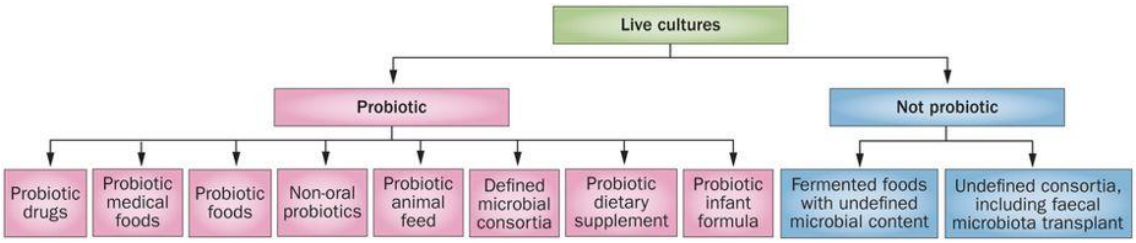


Figure 5. Overall framework for probiotic products. Source: Hill et al. (2015).

The distinction between commensal microorganisms and probiotics is also inferred from this definition. Although commensals in the gut are often the source of probiotic strains, until these strains are isolated, characterized and a credible case presented for their health effects, they cannot be called 'probiotics'. In the years that have passed since the definition was proposed, numerous lines of research have challenged the limits of the probiotic concept, from live cultures present in fermented foods to fecal microbiota transplants (FMT). The term has also been clearly misused, for example, on products such as mattresses, shampoos, disinfectants and aftershave,

for which maintenance of viability and efficacy of the microbes used are not established.

Use of the term probiotic has been restricted in some countries of the European Union because it is deemed misleading to consumers in the absence of approved health claims. It is now evident that different interpretations of the term probiotic are creating notable concerns for major stakeholders with respect to the translation of a large body of research on probiotics to probiotic-containing foods that can benefit consumers. The objectives of the different stakeholders in the probiotic field are described in Figure 6; notably, all the stakeholders' objectives are compatible. Importantly, all parties involved in the probiotic field must work toward a common goal so that society benefits from the scientific advances in the field of probiotic research.

The process that goes from the initial selection of strains to the commercialization of an effective probiotic for a specific target is not easy. Of the thousands of strains isolated each year due to their presumed probiotic potential in laboratories around the world, very few go into a stage of industrial development and much less those that get a hole in the shelves of a pharmacy or food establishment. The main aspects that should be taken into account in the (sometimes, long and winding) path that a strain must follow from its initial isolation until its commercialization are reviewed in the next sections.

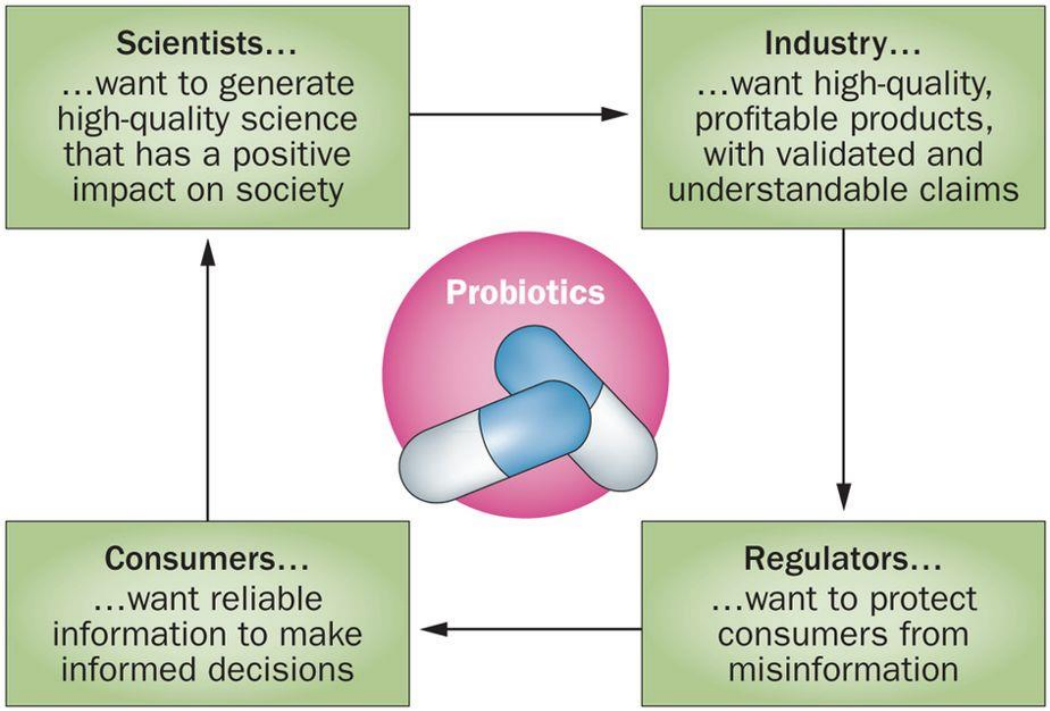


Figure 6. Objectives of stakeholders in the probiotic field. Source: Hill et al. (2015).

II.3.1. Identification of probiotic microorganisms

The identification of an isolate at the species and strain levels is an essential requirement for any isolate that is intended to be commercialized. The allocation of an isolate to one species or another is not banal since the risk assessment according to the European Food Safety Authority (EFSA) is much simpler for those which, based on a history of safe use, enjoy the Qualified Presumption of Safety (QPS) status (EFSA, 2013). The QPS list is reviewed periodically, incorporating new taxonomic units if the available data so guarantee. Consequently, it is likely that in the future, genera and species will be included (e.g.: *Roseburia* spp., *Faecalibacterium prausnitzii*...) that have not been used to date as probiotics but that have been linked with clear effects beneficial for health by several studies on the human microbiome.

Like any other discipline, the taxonomy of bacteria and yeasts is constantly evolving and species determination must be carried out (and, eventually, reevaluated) with the most adequate state-of-the-art methodology. Although phenotypic tests (carbohydrate fermentation, enzymatic activities ...) were very useful when there were no other alternative methods, they are currently not valid for the identification of species since their resolution capacity is clearly insufficient; in fact, the inheritance of the use of inadequate identification methods is the main cause of mislabeling of probiotic products (Huys et al., 2006).

Phenotypic tests were quickly replaced by various molecular techniques, based on fingerprinting or the sequencing of various genes. Among them, partial or complete sequencing of the 16S rRNA gene has practically become the standard method of identification. However, this approach has some drawbacks, such as the existence of non-contrasted sequences in the databases (EMB/GenBank/DDBJ) or its inability to discriminate between closely related (sub)species, such as those that fall within the *Lactobacillus plantarum* group or the *Lactobacillus paracasei* group. In such cases, the use of housekeeping genes, such as *pheS*, *rpoA*, *atpD*, *tuf*, *groEL* or *recA*, or their combination with the 16S rRNA gene, offers a greater discriminatory capacity. In any case, it is unacceptable to prolong the use of obsolete or confusing nomenclature on the labels of the products, although there are examples in this regard in the current probiotic market (eg: *Lactobacillus biphidus* instead of *Bifidobacterium bifidum*; *Lactobacillus sporogenes* instead of *Bacillus coagulans*).

The identification of an isolate at the strain level is equally relevant. The ability to identify a specific strain in a given biological sample or food matrix, or to differentiate it among other probiotics or among the members of the native microbiota of a host, is essential since it enables traceability in laboratory tests, in clinical trials, in epidemiological studies (including possible involvement in adverse effects) and throughout the production and marketing process. On the other hand, it is usual for the company interested in a strain to want to protect it by means of a patent that covers its

possible applications. To do this, the company will have to deposit the strain in a reference collection, such as the Spanish Type Culture Collection (CECT), under the conditions of the Budapest Treaty. In this case, the ability to differentiate the strain is a useful tool to detect possible illegal use by third parties.

Identification at the strain level is also desirable if there are beneficial effects specifically associated with a specific strain. In many documents it is considered that the health effects demonstrated for a specific microbial strain can not be extrapolated or attributable to other strains of the same species; However, this is an aspect that should be reviewed since some effects (and the mechanisms that sustain them) are widely distributed among species belonging to different genera (production of organic acids, competitive exclusion of pathogens ...), others are frequent among the different strains of the same species and, finally, others are more rare (neurological or endocrinological effects ...) and can only be associated with a few strains within a given species (Hill et al., 2014) (Figure 7).

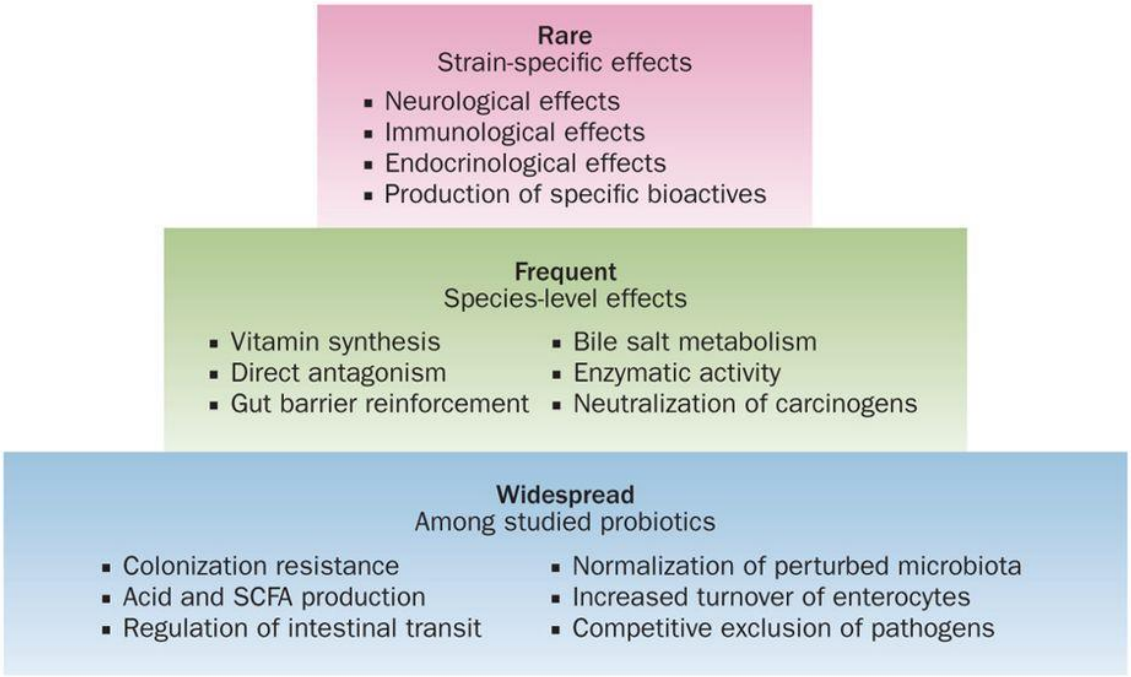


Figure 7. Possible distribution of mechanisms among probiotics at the strain or species levels. Source: Hill et al. (2014).

Despite the availability of various genotyping techniques, pulsed-field gel electrophoresis (PFGE) is still considered the one of choice to differentiate strains, without forgetting that the presence of plasmids and megaplasmids may be associated with properties that differentiate a strain against to similar ones of the same species. The complete genome sequence (including the extrachromosomal elements) is the best possible information for the identification of a species and strain, as well as providing very valuable information about its safety, functionality and technological properties. At

present, functional genomics already facilitates the selection of strains for specific applications and will be a common approach in the near future (Douillard and de Vos, 2014).

II.3.2. Strain safety

The evaluation of the safety of probiotics is obviously an essential requirement in the selection process that begins, as mentioned above, with a correct taxonomic identification. The microorganisms used as probiotics include yeasts (*Saccharomyces cerevisiae*) and bacteria from different genera (*Lactobacillus*, *Streptococcus*, *Enterococcus*, *Pediococcus*, *Bifidobacterium*, *Propionibacterium*, *Bacillus*, *Escherichia*), some of which are part of the native microbiota of the hosts to which they are directed and others not. On the other hand, probiotics have been used in a wide range of situations, including healthy people, healthy people but in a special situation (babies, pregnant or lactating women, elderly...) and people with different types of pathologies and severities. Consequently, the safety assessment must take into account, among other factors, the microorganism in question, the form of administration, the level of exposure, the health status of the host and the physiological functions that they can perform (Sanders et al., 2010).

The cases in which it has been possible to establish a relationship between the consumption of a probiotic and an adverse effect are very scarce and have affected people with serious underlying diseases or with an altered intestinal barrier. This low epidemiological incidence is especially noteworthy considering the wide use of this type of products (Cannon et al., 2005; Boyle et al., 2006). For example, the risk of infection by *Lactobacillus* is approximately one case per 10 million people while, in general, the risk of lactobacilemia is considered as "*unequivocally insignificant*" (Salminen et al., 2002; Borriello et al., 2003; Bernardeau et al., 2008). In such cases, the species most implicated to date have been *Lactobacillus rhamnosus* (Rautio et al., 1999; Mackay et al., 1999), *Saccharomyces cerevisiae* var. *boulardii* (usually associated with the presence of central venous catheters) (Hennequin et al., 2000), and *Bacillus subtilis* (Oggioni et al., 1998; Spinosa et al., 2000).

The fact that no side effects have been observed (and indeed beneficial effects) in the vast majority of clinical trials in which probiotics have been administered to immunocompromised hosts, including people positive for human immunodeficiency virus (HIV), very low or extremely low weight preterm infants of elderly patients, confirms the low pathogenicity of probiotics, even in situations favorable for infections by opportunistic microorganisms (Bernardeau et al., 2006; Trois et al., 2008; AlFaleh and Anabrees, 2013; Moles et al., 2015)

Despite this, the issue of safety is essential in people with serious diseases, especially after observing that the administration of a multi-species probiotic to patients with acute pancreatitis was associated with a higher mortality than the control group

(16% vs. 6%) (Besselink et al., 2008). An analysis of patient subgroups revealed that the probiotic had beneficial effects in moderately ill patients but negative in critical ones. No translocation of any of the strains contained in the probiotic product was observed in the systemic circulation and everything seems to indicate that the adverse effects (higher rate of intestinal ischemia and mortality) were due to an excess of oxidative load on a redox system that was already very altered, causing an increase in damage induced by oxidative stress and ischemia (Ammori, 2003).

In theory, probiotics could produce four types of adverse effects: (1) infectivity or pathogenicity; (2) production of undesirable metabolites; (3) excessive immunostimulation or immunosuppression in sensitive individuals; and (4) possibility of transmission of genes that confer resistance to antibiotics.

II.3.2.1. Pathogenicity

To date, no gene has been unequivocally related to pathogenicity in *Lactobacillus* or *Bifidobacterium* genera, including isolates associated with sepsis or other adverse effects (Vesterlund et al., 2007). The factors that have been proposed to explain their involvement in infections include their adhesion to the host cells (which would facilitate its translocation), the degradation of mucins (which provides metabolites for its growth), the hydrolase activity of bile salts (which facilitates its survival in the intestinal environment) or the resistance to the innate defense mechanisms. However, these are characteristics that, in general, contribute to the colonization of mucosal surfaces and, as such, are shared by a large part of the natural microbiota.

The process of infection and invasion involves a first contact or adhesion between the bacterial cells and the epithelial cells, a translocation through the intestinal epithelium, and an indiscriminate proliferation that can end up in a liver abscess, an endocarditis or a sepsis. For this reason, it has been suggested that the potential for adhesion and translocation of a strain may be a part of the safety assessment of probiotics. However, these are clearly controversial issues. In fact, adhesion capacity has been considered as a possible probiotic characteristic since it can promote the colonization of the target epithelium, the improvement of neuro-immunological interactions, the occupation of receptors (to the detriment of pathogens), and the strengthening of the intestinal barrier (Huys et al., 2013).

Translocation also has numerous nuances; on the one hand, there is an "infectious" bacterial translocation, which implies a high translocation rate, which is usually associated with pathogenic bacteria (enteroinvasive strains of *E. coli, L. monocytogenes* ...), which usually coincides with an alteration of the intestinal barrier and/or of the immune system, and which leads to a pathological situation; on the other hand, "controlled" translocation constitutes a selective and highly regulated physiological process that happens continuously in healthy individuals, which implies a low rate of translocation and is fundamental for the homeostasis of an individual (Rodríguez, 2014).

Therefore, the fact that a strain is capable of translocating in a controlled manner is not only a reason to reject a strain but could even constitute a relevant probiotic property.

In any case, there are many doubts about the validity of *in vitro* tests to predict the adhesion and translocation *in vivo* of a strain since, depending on the physiological state of the bacteria, the different types of tests, the different cell lines used and the differences between laboratories, the results for the same strain can vary considerably.

In contrast to lactobacilli and bifidobacteria, numerous virulence factors (hemolysin, gelatinase, DNase ...) have been described in the genus *Enterococcus*, and especially in the species *Enterococcus faecalis* and *Enterococcus faecium* (Eaton and Gasson, 2001), although there is no consensus on a procedure or method that differentiates the pathogenic from the non-pathogenic strains. Currently, they are not included in the QPS list so, like other microorganisms in the same situation, the evaluation of their safety should be done demonstrating the absence of virulence factors in a strain by strain basis. In the case of the *Bacillus* species used as probiotics and included in the QPS listing (eg: *B. coagulans*, *B. pumilus*, *B. subtilis*), the absence of toxigenic activity (absence of *Hbl* and *Nhe* genes and of cytotoxicity) is required (Duc et al., 2004; ISAPP, 2003).

II.3.2.2. Production of D-lactate

In the past, some authorities considered that the production of the D (-) isomer of lactic acid was a property to be taken into account for the evaluation of the safety of probiotics, especially for use in children. However, from the scientific point of view, it is a property practically irrelevant to determine the safety of an isolate since many of the native species of the human gastrointestinal tract, including some widely used as probiotics in pediatrics (eg: *Lactobacillus reuteri*), produce D-lactic acid. It is also widely accepted that the ingestion of yogurt, which contains high concentrations of this isomer due to the activity of *Lactobacillus delbrueckii* subsp. *bulgaricus*, does not pose a risk to children's health. In this sense, everything seems to indicate that there is no reason to avoid the use as probiotics of human autochthonous lactobacilli on the basis of the lactic acid stereoisomers that they produce (ISAPP, 2003). In fact, to date, no case of acidosis has been described by D-lactic acid in any healthy human being, regardless of age. Clinical trials, in which probiotic D-lactate-producing strains have been administered to full-term and premature infants, have revealed no signs of acidosis, even after daily administration during the first 12 months of life (Connolly and Lönnerdal, 2004; Connolly et al., 2005).

II.3.2.3. Production of biogenic amines

Biogenic amines (BA) are low molecular weight nitrogen compounds that are formed mainly by decarboxylation of amino acids and that exert essential physiological functions for living beings. However, the decarboxylation of some amino acids, carried

out by certain microorganisms, can cause the presence of high concentrations of BA (histamine, tyramine ...) in food. For this to happen, it is important that the raw material contains a high protein load and that adequate conditions for intense proteolysis are given. These conditions occur in certain fermented foods when the fermentation is directed by lactic bacteria with aminoacyl-decarboxylase activity, a property that depends on the strain and not on the species (Fernández and Álvarez, 2005).

It should be noted that there are people especially sensitive to BA because the enzymes responsible for its detoxification, monoamine oxidase (MAO) or diamino oxidase (DAO), are not functional, either because of genetic problems or because of the presence of inhibitors, such as alcohol or some antidepressant drugs. Therefore, it is difficult to establish the toxic levels for each of the BAs since it depends on the effectiveness of the detoxification systems and, therefore, varies from one individual to another. For this reason, the inability to synthesize BA must be included in the selection criteria of starter cultures and also of those probiotics that are going to be transported through food where the conditions for their formation can be foreseen.

II.3.2.4. Resistance to antibiotics

The rise of antibiotic (multi)resistant bacteria represents a serious threat to Public Health. Consequently, the possible presence of transmissible genes that can confer this phenotype to the bacteria of the host's microbiota (including those potentially pathogenic) is a very relevant aspect in the evaluation of the safety of the bacteria that are to be used as probiotics. However, it should be noted that no *in vivo* assay demonstrating the transfer of this type of genes from a probiotic strain to any member of the indigenous autochthonous microbiota has been published up to date.

The determination of antibiotic resistance seems a relatively simple test but, traditionally, trials involving bifidobacteria and lactic acid bacteria (except enterococci) have faced standardization difficulties. It is currently considered that it should be performed according to internationally accepted procedures such as, for example, the most up-to-date version of the Clinical and Laboratory Standards Institute (CLSI) guide. The criteria proposed by Klare et al. (2005, 2007) for this type of bacteria are also widely used.

Antibiotic resistance is part of the safety evaluation scheme proposed by EFSA to determine the QPS status of a strain (EFSA, 2012). To do this, EFSA indicates the antibiotics that should be considered depending on the genus or species to be evaluated and provides cut off values. With some exceptions, this approach requires the determination of the minimum inhibitory concentration (MIC) against ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and/or chloramphenicol. When a strain is resistant to an antibiotic, the type of resistance must be determined. There is “intrinsic resistance” when this property is inherent to all strains of a species while it is “acquired” when a strain of a species

typically sensitive to an antibiotic shows resistance to it. In this second case, it is necessary to present genetic data that explain the cause of the resistance since it can be due to mutations in endogenous genes or by acquisition of exogenous transmissible genes (*tetW*, *tetM*, *tetS*, *ermB* ...) (Ammor et al., 2007; Devirgiliis et al., 2011; van Reenen and Dicks, 2011)

In this context, the criterion of the EFSA (2012) is as follows: (a) the horizontal transmission potential of the intrinsic resistances is minimal, so bacteria that carry them can be used in food; (b) the potential for horizontal transmission of the acquired resistances due to chromosomal mutations is low so that, in general, such bacteria can also be used in foods; (c) bacteria that carry acquired resistance as a consequence of the incorporation of genetic elements (and, especially, mobile genetic elements: plasmids, transposons ...) are those that have the greatest potential of horizontal transmission, so they should not be used in foods; (d) avoid using any strain if genetic information about the nature of a demonstrated resistance is not available; and (e) the presence of resistances does not convert a lactobacillus or a bifidobacterium strain into a pathogenic microorganism.

II.3.2.5. Negative effects on the immune system

The safety assessment of probiotics should also consider their impact on immunologically immature or immunocompromised hosts. All microorganisms, both autochthonous and allogenic (http://www.sepyp.es/es/wiki), exert an impact on the immune system. The autochthonous microbiota is crucial for the development and maintenance of the physiology and homeostasis of the mucous membranes and epithelia that they inhabit, places that act as a highly selective barrier and organ of communication between the luminal environment and the host (O'Hara and Shanahan, 2006); in fact, a failure in this interaction it can contribute to the development of inflammatory, metabolic or infectious pathologies.

In this context, the fact that, in many cases, the mechanisms by which probiotics exert their action are still not well known is a drawback to predict the immunological safety of a probiotic intervention. Whether a probiotic exerts immunostimulatory or immunosuppressive effects (which may be protective or harmful) depends on the interactions between the microbial signals, the genetic basis of the host and the environmental conditions. Several studies have shown that certain probiotic bacteria stimulate the proliferation and activity of immune cells, increasing the effectiveness of the response to pathogens. In contrast, other probiotics are effective against chronic inflammations and allergies through the suppression of effector cells and the induction of tolerance mechanisms (Kalliomaki et al., 2003; Kruis et al., 2004). Knowledge of the relationships between structure (genotype, phenotype) and the functions of probiotics in target populations will limit the risk of inducing adverse immunological effects in a host.

II.3.2.6. Safety of excipients

The safety assessment of a probiotic should take into account the excipients used in the formulation of the final products. As an example, cases of children who have suffered anaphylactic reactions due to exposure to cow's milk proteins used as excipients have been described (Moneret-Vautrin et al., 2006; Lee et al., 2007) In this sense, probiotic products must comply with the regulations in force regarding the declaration of allergens in the labeling. With regard to clinical trials, it is important to include allergy or excipient intolerance among the exclusion criteria.

II.3.2.7. Tests and models for the evaluation of safety

There are numerous types of *in vitro*, *ex vivo* and *in vivo* assays to assess the safety of probiotics. In general, all are useful to have more information when selecting the safest strains, although those based on simple phenotypic tests and the use of cell cultures have, to a greater or lesser extent, the disadvantage of not adequately reflecting the complex interactions that are established in a living being. Animal testing has traditionally been considered an essential part of evaluating the safety of anything that is intended to be administered to humans. For this purpose, a wide range of species belonging to different zoological classes have been used, highlighting, among all of them, the rat model. However, animal models are also subject to controversy because of the inherent ethical connotations and because frequently the safety data obtained are not directly extrapolated to the human species (Sanders et al., 2010) Therefore, it is desirable that the animal model be selected for its ability to predict what may happen in a person, which implies that their anatomy and physiology, including their development, metabolic processes, immune system responses and the composition of the microbiome, are as similar as possible to ours. In this sense, the pig is the ideal model (Pang et al., 2007), despite the fact that it poses problems of cost and space, which often make it necessary to opt for other species.

To date, studies of acute, subchronic and chronic oral toxicity of probiotics in standard models (eg, healthy rats) have shown no adverse effects even when administered at high doses (up to 10,000 times greater) than those normally consumed in humans) during a prolonged period of time (Ishibashi and Yamazaki, 2001; Reid et al., 2003; Sanders et al., 2010). Due to this low or no pathogenicity, some authors have resorted to the use of animals which are immunosuppressed or genetically predisposed to suffer from certain pathologies in order to evaluate the safety of probiotics although this strategy has also been questioned because of the difficulty of extrapolating the results to a real situation. Another alternative that has been used to try to force the pathogenicity of probiotics is its administration by unusual routes (intravenous, intraperitoneal ...). Again, these approaches are difficult to validate since the results obtained can not be extrapolated to what happens when the same strain is administered orally (Ishibashi and Yamazaki, 2001). Globally, although the different *in vitro*, *ex vivo*

and animal models assays can provide useful information during the process of selection of strains, the only data that allow assessing the safety of a probiotic in a direct way are those obtained in the course of phase 1, 2 and 3 human clinical trials, properly designed and specifically directed to the target population.

II.3.3. Functionality

Similar to the safety assessment, there are also numerous *in vitro*, *ex vivo* and *in vivo* tests to detect those strains that have relevant functional properties. To a greater or lesser extent, all are useful to have as much information as possible when scrutinizing the strains with the greatest probiotic potential, but in practice, most of the *in vitro* and *ex vivo* tests do not allow guarantee the functionality of probiotic microorganisms in a host. Again, it will be clinical trials (phase 2 and 3) that determine whether a probiotic exerts the beneficial effect that was expected on the target population (Holzapfel et al., 1998).

From the functional point of view, the selection criteria usually include, on the one hand, a series of prerequisites for the strain to reach its place of action at an adequate concentration (normal inhabitant of the target host, resistance to transit through the digestive system, capacity of adhesion to epithelial cells ...) and, on the other hand, properties that could be associated with a beneficial effect in a host (production of antimicrobial substances, competitive exclusion of pathogens, stimulation of mucin synthesis, production of short chain fatty acids, synthesis of specific bioactive compounds, neutralization or detoxification of carcinogens and abiotic contaminants, immunomodulation, endocrinomodulation, neuromodulation ...). Among the last type of properties, some mechanisms might be widespread among commonly studied probiotic genera; others might be frequently observed among most strains of a probiotic species; others may be rare and present in only a few strains of a given species. Evidence is accumulating on a cross-section of probiotic strains that suggest some generalizations can be made beyond strain-specific effects (Figure 7).

II.3.3.1. Prerequisites

Traditionally it was recommended that probiotic strains had to be originally isolated from samples of the same species to which they were to be administered, based on the belief that strains of human origin would implant or colonize the human gastrointestinal epithelium more easily than strains isolated from samples belonging to other species (Holzapfel et al., 1998; Ouwehand et al., 1999). However, the report of the aforementioned FAO/WHO working group concluded that there is no defined criterion for the term "*human origin*" (FAO/WHO, 2002) since, in many cases, it is extraordinarily difficult, if not impossible, know the ultimate origin of a strain (human, animal, vegetable, food ...) even though it has been isolated, for example, from a sample of human feces. It also pointed out that there are several examples of probiotic strains that belong to allochthonous species but with beneficial effects well documented in

humans. For all these reasons, it concluded that, globally, the property "*human origin*" does not constitute a relevant criterion in the selection of probiotics for its use in our species.

In order for orally administered probiotic strains to exert their beneficial effects, they must be able to withstand the environmental conditions existing during transit through the digestive system (saliva, gastric acidity, bile, pancreatic secretion ...), taking into account that the composition of the different secretions, the time of gastric emptying or the intestinal motility can vary depending on the age and health status of the host. To determine the resistance, *in vitro* methods, such as acidification and/or the addition of bile salts to the culture medium, the use of gastrointestinal secretions obtained from healthy individuals or the use of more sophisticated dynamic models such as those developed by the TNO in the Netherlands (Marteau et al., 1997), have been proposed. The survival of probiotics during transit can also be studied *in vivo* using intestinal intubation techniques and biopsies of the colon or analyzing their presence in the feces of people or animals that have ingested them, resorting to molecular techniques that allow discriminate between members of the host's indigenous microbiota.

An important factor for the survival of probiotic bacteria is the substrate or matrix with which they are transported and, in fact, the results obtained in culture media can not be extrapolated to what happens when the strains are transported through food or encapsulated (Saxelin et al., 1993; Saxelin et al., 1995; Charteris et al., 1998). Systems that allow the maximum protection of probiotic strains during their passage through the stomach and duodenum have been developed in the last years, so that this criterion does not imply a real limitation. The same is applicable for the administration of probiotics by other classical (vaginal) or emerging routes (ophthalmologic eye drops, ear solutions, skin applications,..) in relation to the specific conditions of each ecosystem.

II.3.3.2. Probiotic properties

The functional properties by which a probiotic is selected can be as broad as our imagination, technology or budget can allow. For this reason, the intention of this section is not to offer a complete catalog of potentially probiotic characteristics but to highlight the diversity and complexity of the existing alternatives. Ideally, it would be necessary to know what a probiotic is desired for and what population it is intended to apply; in this way, they would be selected through the most appropriate tests to highlight the properties that are considered most relevant to achieve the final objective.

As discussed above, adhesion to epithelial cells is a controversial property. However, for many authors it remains a key characteristic for a strain to colonize a mucosa and exert a probiotic effect, which can range from the competitive exclusion of pathogens (by competing for the same receptors) to complex neuroimmunological interactions, through induction of the biosynthesis of antimicrobial peptides or mucins by the epithelial cells. Various surface proteins, including mucus-binding proteins, and

some specific structures, such as the pili described in some strains of *L. rhamnosus*, are involved in the adhesion process. Self-aggregation phenomena can substantially increase the colonization capacity in those ecosystems in which probiotics have a short residence time. Another relevant property of some probiotic strains is to coaggregate with certain pathogens and, consequently, prevent their access to mucous membranes. The antimicrobial effect of the congregation is particularly intense when the same strain is capable of producing antimicrobial substances (organic acid, hydrogen peroxide, bacteriocins, reuterin ...) that inhibit the pathogen in question (Boris et al., 1998).

The integrity of the mucous membranes is influenced by many factors, including changes in permeability, mucin composition, oxidative stress or the relationship between the production of new epithelial cells and the rate of apoptosis of damaged or aged epithelial cells. Several studies have demonstrated the ability of some probiotics to maintain or improve intestinal barrier function by modifying the expression of proteins that are part of the occlusion zones (occludin ZO-1, claudin-1, claudin-4,...), the modification of the monosaccharide composition of the mucins, the increase in the thickness of the mucus layer, the inhibition of apoptosis processes and/or the promotion of cell differentiation and cytoprotective activities, including reduction of oxidative stress (Howarth and Wang, 2013).

The lymphoid tissue associated with the mucous membranes represents the majority of the immune system and its interaction with the microbiota constitutes one of the pillars of health. In fact, the processes of dysbiosis of the endogenous microbiota alter the immune responses and contribute to the appearance of infectious, inflammatory and (auto)immune diseases. Therefore, it is not surprising that the immunomodulation capacity is one of the activities that have been most associated with probiotics. In this regard, the effect of various strains on the different components of both innate (NK cells, dendritic cells, macrophages, epithelial cells...) and adaptive or acquired (Th1, Th2, Th17, Treg, Tc and B) immunity, including the proliferation and gene expression of various populations of cells of the immune system and the production of a broad spectrum of immunoglobulins, cytokines, chemokines, and growth factors has been repeatedly reported (Hardy et al., 2013).

However, the selection process must take into account that the type of immune response associated with a probiotic (immunoactivation, immunodeviation, immunoregulation, immunosuppression) can be positive or negative depending on the state of the host. Consequently, to modulate an immunopathy for the benefit of the host, one must have as much knowledge as possible about the mechanisms responsible for the pathology and about the responses that can be expected from the probiotic. Therefore, the strain must be carefully selected depending on the population to which it is directed.

Neuromodulation is one of the most promising effects in the field of probiotics. The human gastrointestinal tract contains a very complex nerve network, called the

enteric nervous system, whose main objective is the regulation of physiological functions and the modulation of communication between the intestine and the central nervous system, both upstream (bowel-brain) and downstream (brain-gut) (Mayer, 2011). This communication system is known as the "*gut-brain axis*" and serves as coordination between the brain, the gastrointestinal tract, the endocrinological system and the immune system. Alterations in the bowel-brain axis are usually associated with certain psychiatric (from anxiety and depression to autism) and intestinal (irritable bowel syndrome) pathologies and the presence of an aberrant microbiota in individuals who suffer them (Critchfield et al., 2011; Saulnier et al., 2013). In this sense, it is considered that probiotics can have an important impact for these populations although, again, it will be necessary a careful selection of the strains (interaction with nervous receptors, effects in the biosynthesis and metabolism of neurotransmitters,...) and studies that reveal the magnitude, mechanisms and clinical relevance of the possible beneficial effects.

There is a broad and growing spectrum of phenotypic tests to manifest probiotic properties through *in vitro* procedures. In addition, the availability of a functional genome analysis using new generation sequencing technologies and the rest of the –omics approaches have revolutionized the discovery of potentially probiotic properties within a strain (Saulnier et al., 2011; Tu et al., 2014). Such powerful techniques allow a detailed analysis of genes involved in colonization, persistence, interaction and signaling within the human host and, consequently, a rapid selection of strains with very specific properties.

II.3.3.3. In vivo assays

Currently, *in vivo* assays offer the advantage of being able to apply a wide range of techniques (from –omics to the advanced imaging techniques, including biochemical techniques, molecular microbiology and cell biology) to try to elucidate the mechanisms of action and identify markers related to the beneficial effects (and, eventually, the possible adverse effects) of a probiotic.

The use of animal models allows the study of samples, tissues and organs that, for ethical reasons, it is impossible to access in human clinical trials, so they are still essential to determine action mechanisms and biomedical markers. The range of animal models used to date to demonstrate the functionality of probiotics *in vivo* is very broad, both in animal species (including nematodes, insects, amphibians, fish, birds and mammals) and in types of animals within a specific species (breeds, pathophysiological states, germ-free animals, animals with a microbiota or "humanized" immune system, knock-out animals,...).

Despite its undoubted usefulness, there are many anatomical and physiological differences (metabolic processes, immunological and endocrine responses, composition of the microbiome ...) between animal models and the human species; therefore, it will

be the human clinical trials the ones that finally determine the efficacy of the probiotic for the chosen target. Previously, descriptive human studies comparing samples of healthy people and people with different pathologies are extremely useful in determining the markers to be included in clinical trials. The definition of probiotic contains the word "benefit" and, in this sense, the objective of clinical trials is to determine scientifically if it actually occurs and its magnitude.

The clinical trials in which efficacy is evaluated are typically those of phase 2 and phase 3. The phase 2 studies evaluate the efficacy of a probiotic against a placebo, preferably in a double-blind format, and collect the possible adverse effects. The desirable result would be a biological and statistically significant improvement in one or more of the following aspects: well-being or quality of life, reduction in disease risk, faster recovery from a disease, milder symptomatology during a disease and/or increase in recurrence times. More clinical evidence derived from this type of studies is needed for probiotics (species, strain, formulation, dose, specific application for which they have shown efficacy) to gain credibility among consumers and, especially, among the medical community, regardless of whether or not are marketed in the form of food or drug presentations.

Phase 3 studies evaluate the efficacy of a probiotic versus the standard therapy used to prevent or treat a particular disease (Arroyo et al., 2010). In general, they are randomized trials in which the sample size must be carefully calculated and they should include possible adverse effects and incidents, an assessment of the risk: benefit ratio and a series of controls to check the quality of the trial. It would be desirable to carry out more phase 3 studies to encourage the use of probiotics in the prevention and treatment of diseases in those cases in which they can replace or complement conventional medicines. Currently, there are several factors that make more and more doctors consider the possibility of using probiotics as an alternative to certain medications, among which the growing number of (multi)resistance to antibiotics among pathogenic microorganisms is probably the most important one.

The FAO and WHO working group recommended publication in internationally recognized scientific or medical journals, both for documentation proving the probiotic nature of a strain (including evidence from clinical trials) and for those cases in which negative results are obtained (FAO/WHO, 2002)

II.3.4. Technological aspects

The fact that a bacterial strain grows well under laboratory conditions (small volumes, complex culture media...) does not mean that the same will happen in industrial conditions. In this sense, companies that commercialize or wish to commercialize probiotics face two important technological challenges: (1) the need to obtain a very high bacterial biomass in an economically viable way; and (2) the need for the

concentration of bacteria necessary to exert the beneficial effect to remain viable until the end of the shelf life of the product (Rodríguez, 2015).

Both aspects are related to the physiological characteristics of each strain, so conditions must be established case by case. In addition, viability also depends on the format in which the bacteria are to be administered since, for example, the shelf life of refrigerated dairy probiotic products is significantly shorter than that of lyophilized products sold with a medicinal presentation (capsules, powder ...). At the same time, there are several parameters (concentration of oxygen, humidity, storage temperature ...) and formats (microencapsulation, coatings ...) that play an important role in the stability of the product. In any case, it is inevitable that a greater or lesser rate of bacteria will die or be damaged during the productive process or the storage of the probiotic product and, in this sense, companies usually resort to the initial overdose of the probiotic, in such a way that keep the effective dose at the end of its useful life.

Companies that are dedicated to the development of probiotics (including industrial scaling and feasibility studies) usually organize their work in the form of stages with an increasing degree of difficulty in which it is absolutely necessary that the objectives of a phase be achieved in order to be able to move on to the next one. The first stage usually consists of: (a) the deposit of the stock in the bank of the company and the verification of its identity (species); and (b) evaluation of its fermentative capacity (production, ropyness, morphology ...) on a small scale in mini-bioreactors or mini-fermenters that simulate the conditions of the plant fermenters (temperature, pH, agitation ...).

The second phase involves scaling to pilot plant productions to evaluate productivity before and after the lyophilization process and the study of stability after three months. In general, the objective is for the product to be stable (loss ≤ 0.2 log cfu) in a mixture of cellulose (or other excipient) packed in foil pouches stored at a constant water activity (<0.2) and at a temperature of 25 °C. In the next phase, the workflow is determined so that the probiotic enters into the production phase. After the first production, the price is defined on the basis of the product profile (cfu/g) and the analytical validation of the mixture is completed. The final goal is the release of the product while long-term stability studies (two years) continue, both in refrigeration and at room temperature (~25ºC). In general, it is intended that the production process allows having a product with a high concentration ($> 5 \times 10^{10}$ cfu/g) which, once dosed in the final containers, has a prolonged useful life at room temperature (>2 years). In cases where it is not possible, the product must be kept refrigerated until its sale.

In any case, it is essential to apply the principles of the HACCP system (Hazard Analysis and Critical Control Points) and good manufacturing practices to ensure that probiotic products reach the consumer with a quality as highest as possible. The quality control should consider the presence of the strains at the appropriate concentration, their

viability and stability and the possible contamination of the final product with other microorganisms, using the most appropriate dependent and independent culture techniques in each case (Huys et al., 2013). It is advisable that the companies that commercialize probiotics make controls of the products of the competition within the framework of the protection of patented strains.

When a company introduces a probiotic into the market, it should ensure that the product is well labeled. In this regard, the expert committee of the FAO/WHO (2012) recommended that the label of any product containing probiotics include the following information: (1) genus, species and strain; (2) minimum dose of viable microorganisms at end of life; (3) necessary amount of product that must be consumed to achieve the effective dose; (4) beneficial effect(s); (5) storage conditions; and (6) information to contact with the customer service.

II.3.5. Commercial aspects

The opinion of the commercial department of a food or pharmaceutical company is essential when making a decision about the industrial development and the eventual placing on the market of a probiotic. The functions of this department include, among others, market studies (which allow detecting those needs of consumers that may be profitable), the promotion and advertising of the product, sales and post-sales service. Normally, these are activities that involve constant interaction with the production, financial and human resources departments.

In general, three figures are usually identified in relation to the purchase decision: the prescriber (professional who recommends the product and whose opinion is valued by the consumer, for example, doctor or pharmacist), the buyer (the person who acquires the product but that does not have to coincide with the consumer, for example, parents who buy a probiotic indicated for infant colic), and the consumer. On the other hand, it is important to know the market segmentation, either by sex (for example, probiotics for vaginal infections or for mastitis), by age (for example, pediatric probiotics), by level of income, etc.

Marketing campaigns basically focus, as in any other type of product, on different elements: the characteristics of the product (including the packaging), its price, distribution or availability and after-sales service. Normally, the life cycle of a product includes the stages of introduction or launching (a new product to the market, sales occur but the balance may become negative since it involves a significant promotion expense), growth (the product begins to be known, sales experience strong growth and profits too), and maturity (the speed of sales growth begins to stabilize and profits do the same). Eventually, there may be a stage of decline or saturation (considerable drop in sales and profits), in which you can try to relaunch the product by introducing some innovation.

The determination of the price can be based on the costs, on the elasticity of the demand, on the prices fixed by the competition for similar products, etc. In general, the probiotic products available in pharmacies and parapharmacies are not covered by the public health system so they usually have a high final cost (~ € 1/capsule, sachet or ovule). This fact prevents a relatively high percentage of potential users to have access to them, especially when prolonged treatment is required. In the future, the greater availability of results based on well-designed clinical trials and certain regulatory changes could change the situation.

The distribution policy allows the product to be in the right place at the right time to be purchased by the consumer. In general, the process that the product follows since it leaves the production chain until it reaches the customer is as follows: product storage, physical distribution, billing and collection. The distribution channel is any of the means (food establishments, pharmacies, parapharmacies ...) that are used to get products to go the way from the producer to the consumer. There are several ways to promote the product, including advertising, sales promotion, public relations and merchandising or advertising at the place of sale.

Finally, the interaction between the researchers and clinicians who isolated and evaluated the probiotic strain(s) and the company that commercializes it is very important, especially in relation to the support that the former can offer in presentations in meetings, congresses and specialized publications or in the form of informative information for a wider audience.

III. OBJECTIVES

S. agalactiae (GBS) is a bacterial specie that is often present in the microbiota of the human gastrointestinal and genitourinary tracts of healthy hosts. The GBS colonization rate oscillates between 15 and 40% among fertile women, with a mean value of 18% worldwide (Russell et al., 2017). However, under certain circumstances *S. agalactiae* can cause infections both in adults, especially pregnant women, immunocompromised people and elderly people, and, particularly, in infants (Le Doare and Heath, 2013). In fact, this microorganism is one of the leading causes of EOS and LOS, which are associated with significant morbidity and mortality. EOS usually reflects transplacental or ascending infections from the maternal genitourinary tract, whereas LOS has been generally linked to the postnatal nosocomial or community environments.

Because of the involvement of *S. agalactiae* in LOS, there is an interest in eradicating this microorganism from the gastrointestinal and genitourinary tracts of pregnant women. In the last decades, intrapartum antibiotic prophylaxis (IAP) has been the most common preventive strategy in developed countries. National strategies for targeting pregnant women for IAP include either risk-based or screening-based approaches. Risk factors include intrapartum fever above 38ºC, premature (before 37 weeks of gestation) or prolonged (>18 h) rupture of membranes, a previous infant affected by GBS disease and *S. agalactiae* bacteriuria (≥10 cfu/ml). On the other hand, universal screening involves the collection of recto-vaginal swab at late pregnancy (35-38 week of pregnancy) and the subsequent administration of IAP to positive-women and to those with an unknown status. However, even strict and universal implementation of IAP guidelines does not eliminate early onset disease by GBS disease while IAP has no impact on late onset GBS infection where the burden of disease is also substantial.

As a method to eradicate GBS neonatal infections, the IAP strategy is not devoid of controversy. Some Cochrane's reviews on this issue have concluded that the efficacy of IAP to reduce perinatal GBS infections are not supported by conclusive evidence from well designed and conducted randomized controlled trials (Ohlsson and Shah, 2009; Ohlsson et al., 2013; Ohlsson et al., 2014). A major drawback of the universal screening strategy is that, as stated above, most women colonized with GBS are asymptomatic and, among GBS-positive women, very few will give birth to babies who are infected with GBS. Hence, giving intravenous antibiotics to all GBS-positive pregnant women during delivery will put a large number of women and babies at risk of the adverse effects of antibiotics unnecessarily. These adverse effects include anaphylaxis and allergic reactions. However, the biggest concerns are the increasing rates of antibiotic resistance among clinically relevant microorganisms and, particularly, the impact of IAP on the acquisition, composition and development of the infant microbiota.

Given the limitations in IAP strategies, alternatives are required. Vaccines, in particular, hold great promise but no GBS vaccine is currently available since vaccine development targeting this bacterial species also faces some relevant limitations. But, even if an effective vaccine to prevent GBS infections is developed in the future, a need for alternative strategies is still likely to be present as all women will not be immunized and the vaccine may not be effective in women giving birth preterm. In this context, the isolation, selection and characterization of safe probiotic strains with the ability to antagonize and, eventually, eradicate GBS from maternal and infant mucosal surfaces is a very attractive target. This has been the general objective of this PhD Thesis.

The partial objectives of the PhD Thesis were the following:

1. Analysis of the bacterial diversity and the presence of GBS in vaginal and fecal samples of pregnant and non-pregnant healthy women. Isolation of lactobacilli strains from the biological samples.

2. Characterization of the preselected lactobacilli strains by *in vitro* assays targeting both potential probiotic properties against GBS and safety (antimicrobial activity against GBS strains, co-aggregation with GBS strains, adhesion to mucin and intestinal and vaginal cells, resistance to the transit through a gastrointestinal tract-like environment, production of biogenic amines, mucin degradation, antibiotic resistance profile, etc).

3. *In vivo* (rat model) assessment of acute and chronic toxicities of the *Lactobacillus* strain selected because of its highest probiotic potential to eradicate GBS on the basis of the activities carried out to achieve the partial objective 2.

4. Clinical trial to evaluate the ability of the selected *Lactobacillus* strain for the eradication of GBS colonization in pregnant women.

IV. MATERIALS AND METHODS

IV.1. ASSESSMENT OF THE VAGINAL MICROBIOTA OF GBS-POSITIVE AND GBS-NEGATIVE NON-PREGNANT AND PREGNANT WOMEN

IV.1.1. Participating women

A total of 54 fertile women (30 non-pregnant and 24 pregnant women), aged 25-35, participated in this study. All volunteers gave written informed consent to the protocol, which had been approved (protocol 10/017-E) by the Ethical Committee of Clinical Research of Hospital Clínico San Carlos, Madrid (Spain). All non-pregnant women provided 4 vaginal exudate samples, which were collected at days 0, 7, 14 and 21 of their menstrual cycles. Pregnant women provided a single sample which was collected at week 35-37 of pregnancy. All women claimed to be completely healthy.

IV.1.2. Microbial isolation, enumeration and identification

Samples were diluted in peptone water and spread onto Columbia Nalidixic Acid (CNA), Mac Conkey (MCK), Sabouraud Dextrose Chloramphenicol (SDC), Gardnerella (GAR) and Mycoplasma agar plates (BioMerieux, Marcy l'Etoile, France) for selective isolation and quantification of the main agents involved in vaginal infections. They were also spread onto agar plates of MRS (Oxoid, Basingstoke, UK) supplemented with either L-cysteine (0.5 g/L) (MRS-C) or horse blood (5%) (MRS-B) for isolation of lactobacilli. All the plates were incubated for 48 h at 37ºC in aerobic conditions, with the exception of the MRS-C and MRS-B ones, which were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) in an anaerobic workstation (DW Scientific, Shipley, UK). Parallel, all the samples were submitted to an enrichment step in Todd Hewitt broth (Oxoid, Basignstoke, UK) to facilitate the isolation of *S. agalactiae* in CNA plates.

Initially, identification of the bacterial strains (at least one isolate of each colony morphology per medium and per sample) was performed by sequencing a 470 pb fragment of the 16S rDNA gene PCR amplified using the primers pbl16 (5'-AGAGTTTGATCCTGGCTCAG-3') and mbl16 (5'-GGCTGCTGGCACGTAGTTAG-3') (Kullen et al., 2005). The PCR conditions were as follows: 96 ºC for 30 s, 48 ºC for 30 s and 72 ºC for 45 s (40 cycles) and a final extension at 72 ºC for 4 min. The amplicons were purified using the Nucleospin® Extract II kit (Macherey-Nagel, Düren, Germany) and sequenced at the Genomics Unit of the Universidad Complutense de Madrid, Spain. The resulting sequences were used to search sequences deposited in the EMBL database using BLAST algorithm and the identity of the isolates was determined on the basis of the highest scores (>99%).

Identification of yeasts and confirmation of the initial 16S rDNA-based bacterial identifications was performed by MALDI-TOF (VITEK MS, BioMerieux). Briefly, a

portion of a bacterial colony (~1 µL) was directly spotted onto a MALDI sample plate. Then, it was overlaid with 1 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile (28%), and allowed to dry at room temperature. For each isolate, a mean spectrum was constructed with at least 50 m/z spectra profiles and used for the identification by comparison with the spectra contained in the Myla database (Biomerieux). Identification was defined as a 99-100% match to the species-specific m/z values in the database. Identification of *S. agalactiae* isolates was also confirmed by using a latex agglutination test (Streptococcal grouping kit, Oxoid), following the instructions of the manufacturer.

Those isolates identified as belonging to the genus *Lactobacillus* were preserved for further studies. For such purpose, a MRS-C broth culture of each isolate was mix with glycerol (15%, v/v) and kept at -80ºC until required. A total of 89 different *Lactobacillus* strains were isolated from the vaginal swabs and submitted to Random Amplification of Polymorphic DNA (RAPD) genotyping in order to avoid duplication of isolates. RAPD profiles were obtained using primer OPL5 (5'-ACGCAGGCAC-3'), as described by Ruiz-Barba et al. (2005). *Lactobacillus* strains were routinely cultured in MRS-C medium at 37 °C under aerobic conditions. When required, they were grown in anaerobiosis as described above.

IV.2. *IN VITRO* ASSESSMENT OF THE ACTIVITY OF THE LACTOBACILLI STRAINS AGAINST GBS AND THEIR POTENTIAL MECHANISMS OF ACTION

IV.2.1. Antimicrobial activity against *S. agalactiae* strains

An overlay method (Magnusson and Schnürer, 2001) was used to determine the ability of the lactobacilli strains to inhibit the growth of 12 different *S. agalactiae* strains. Among them, 6 strains had been isolated from blood or cerebrospinal fluid in clinical cases of neonatal sepsis (Hospital Universitario Ramón y Cajal, Madrid, Spain) while the remaining 6 ones had been isolated from vaginal samples of pregnant women (our own collection). It was performed using MRS agar plates, on which the lactobacilli strains were inoculated as approximately 2 cm-long lines and incubated at 37ºC for 48 h. The plates were then overlaid with the indicator *S. agalactiae* strains vehiculated in 10 ml of Brain Heart Infusion (BHI, Oxoid) broth supplemented with soft agar (0.7%), at a concentration of ~10^4 colony-forming units (cfu)/ml. The overlaid plates were incubated at 37ºC for 48 h and, then, examined for clear zones of inhibition (> 2 mm) around the lactobacilli streaks. All experiments assaying inhibitory activity were performed in triplicate.

IV.2.2. Production of bacteriocins

The *Lactobacillus* strains were grown in MRS broth at 37ºC until early stationary phase (A620 ~1.0). The culture was centrifuged at 12000 × *g* for 10 min at 4ºC, and the supernatant was neutralized to 6.2 with 1 M NaOH, heated at 100ºC for 5 min and filter-sterilized through 0.22 μm-pore-size filters (Millipore, Bedford, USA). The bacteriocinogenic activity of the cell-free supernatants was determined by an agar well diffusion assay. Aliquots (100 μl) of the supernatants were placed in wells (7-mm diameter) cut in cooled BHI agar plates previously seeded (10^5 cfu/ml) with the *S. agalactiae* indicator strains. The plates were kept at 4ºC for 2 h and, then, incubated under optimal conditions for growth of the indicator.

IV.2.3. Production of hydrogen peroxide

Hydrogen peroxide production by the lactobacilli strains was initially tested following the procedure described by Song et al. (1999). MRS agar plates supplemented with 0.25 mg/ml of tetramethylbenzidine (TMB; Sigma, St. Louis, USA) and 0.01 mg/ml of horseradish peroxidase (HRP, Sigma) were inoculated with the strain and anaerobically incubated for 2 days at 37ºC. HRP is known to oxidize TMB in the presence of hydrogen peroxide to form a blue pigment in the H_2O_2-producing colony. Parallel, the hydrogen peroxide was also measured by a modification of the quantitative method of Yap and Gilliland (2000). The strain was anaerobically grown in 10 ml of MRS broth for 24 h at 37ºC. The cells were harvested by centrifugation at 12000 × *g* for 10 min at 4ºC, washed twice with potassium phosphate buffer (50 mM, pH 6) and resuspended in 9 ml of the same buffer supplemented with 5 mM glucose. The cell suspension (0.5 ml) was inoculated into a tube containing 9 ml of the glucose-containing buffer. After an incubation at 37ºC for 24 h in aerobiosis, the cells were removed by centrifugation at 12000 × *g* for 10 min at 4ºC and the supernatants were assayed for hydrogen peroxide. Briefly, 5 ml of supernatant were mixed with 100 μL of 1% aqueous *ø*-dianisidine (Sigma), and 1 ml of 0.001% aqueous HRP. The tubes were incubated for 10 min at 37ºC and the reaction was stopped by adding 0.2 ml of 4 N HCl. Absorbance readings (A_{400} nm) were determined and peroxide content was quantified by comparing the values obtained with those of a H_2O_2 standard curve. In the H_2O_2 assays, *Lactobacillus acidophilus* CECT 903T and *Lactobacillus gasseri* CECT 5714 were used as a positive control.

IV.2.4. Production of lactic acid

Lactic acid production by the *Lactobacillus* strains was determined in MRS broth (pH 6.2). One percent inoculum's from an overnight MRS culture was used and incubation proceeded for 24 h at 37ºC anaerobically. Cells were removed by centrifugation at 12000 × *g* for 5 min and the concentration of L- and D-lactic acid in the supernatants was quantified using an enzymatic kit (Roche Diagnostics, Mannheim,

Germany), following the manufacturer's instructions. The pH values of the supernatants were also measured. All these assays were performed in triplicate and the values were expressed as the mean ± SD. *L. salivarius* CECT 5713 was used as a positive control (high production of L-lactic acid in MRS broth) (Martín et al., 2006).

IV.2.5. Co-aggregation between the lactobacilli and the *S. agalactiae* strains

Coaggregation assays were designed based on previously reported methods (Reid et al., 1990). Bacterial suspensions were adjusted to an A_{600} of 0.6. Aliquots of 10 ml of each of the *Lactobacillus* strains were independently mixed with 10 ml of each of the *S. agalactiae* strains and incubated at 37°C for 4 h and 16 h. The suspensions were then observed under a phase-contrast microscope after Gram staining.

IV.2.6. Broth co-cultures of the lactobacilli and the *S. agalactiae* strains

To test the anti-*S. agalactiae* activity of the lactobacilli in a broth assay format, tubes containing 20 ml of MRS broth were co-inoculated with 1 ml of a *Lactobacillus* strain culture (7 $\log_{10}$ cfu/ml) and 1 ml of a *S. agalactiae* strain (7 $\log_{10}$ cfu/ml). Subsequently, the cultures were incubated for 6 h at 37°C in aerobic conditions. Immediately after the co-inoculation and after the incubation period, aliquots were collected, serially diluted and plated on MRS-C plates and CHROMagar StrepB agar plates (CHROMagar, París, France) for selective enumeration of lactobacilli and streptococci, respectively. Correct taxonomic assignment was confirmed by MALDI-TOF analysis as described previously.

IV.3. *IN VITRO* ASSESSMENT OF OTHER PROBIOTIC PROPERTIES OR PRERREQUISITES OF THE LACTOBACILLI STRAINS

IV.3.1. Survival after transit through an *in vitro* gastrointestinal model

The survival of the strains was tested in an in vitro model of the human stomach and small intestine based on that described by Marteau et al. (1997) with the modifications included by Martín et al. (2005). Aliquots (25 ml) containing approximately 10^9 cfu/ml of the strain tested was diluted in 5 ml of a sterile electrolyte solution containing 6.2 g/l of NaCl, 2.2 g/l of KCl, 0.22 g/l of CaCl2, and 1.2 g/l of $NaHCO_3$ to simulate the *in vivo* dilution by host saliva. Then, 5 ml of porcine gastric juice was added and the mixture was incubated at 37ºC with agitation. The pH curve in the stomach-resembling compartment was controlled to reproduce the values found in monogastrics after yogurt consumption (Conway et al., 1987): pH 5.0 at initiation, pH 4.1 at 20 min, pH 3.0 at 40 min, and pH 2.1 at 60 min.

Fractions were successively taken from this compartment at 20, 40, 60, and 80 min, in a manner that simulates the normal gastric emptying (Marteau et al. 1997). After adjusting their pH to 6.5 ± 0.2 with 1 M $NaHCO_3$, they were mixed with 10 ml of a

sterile electrolyte solution containing 5 g/l of NaCl, 0.6 g/l of KCl, 0.3 g/l of $CaCl_2$, 4% of porcine bile, and 7% of pancreatin (Sigma), which simulates the content of the duodenal juice. After 120 min of successive exposure to these conditions, bacterial survival was determined by plating the samples onto MRS agar plates, which were anaerobically incubated at 37ºC for 48 h. *L. salivarius* CELA2 was used as a positive control because of its high resistance to gastrointestinal-like conditions (Martín et al., 2009). All these assays were performed in quadruplicate and the values were expressed as the mean ± SD.

IV.3.2. Adherence assays to intestinal and vaginal epithelial cells

The adherence of lactobacilli to HT-29 and Caco-2 cells was examined as described by Coconnier et al. (1992). Routinely, cells were grown in DMEM medium (PAA, Linz, Austria) containing 25 mM glucose, 1 mM sodium pyruvate and supplemented with 10% heat-inactivated (30 min, 56ºC) fetal calf serum, 2 mM L-glutamine, 1% non-essential amino acid preparation, 100 U/ml penicillin and 100 mg/ml streptomycin. For the adherence assays, HT-29 and Caco-2 were cultured to confluence in 2 ml of medium devoid of antibiotics. Approximately 10 days post-confluence, 1 ml of the medium was replaced with 1 ml of *Lactobacillus* suspension (10^8 cfu/ml in DMEM). The inoculated cultures were incubated for 1 h at 37ºC in 5% CO_2. Then, the monolayer was washed five times with sterile PBS, fixed with methanol, stained with Gram stain and examined microscopically. The adherent lactobacilli in 20 random microscopic fields were counted for each test. *L. rhamnosus* GG and *Lactobacillus casei* imunitass were used as positive and negative controls because of their high and low adhesive potential, respectively (Martín et al., 2005).

Adherence to vaginal epithelial cells collected from healthy premenopausal women was performed as described previously (Boris et al., 1998).

IV.3.3. Adherence to and/or degradation of mucin

The adhesion of the lactobacilli strains to mucin was determined according to the method described by Cohen and Laux (1995) with some modifications. Briefly, 100 µl of a solution (1 mg/ml) of porcine mucin (Sigma) in HEPES-buffered Hanks salt solution (HH) were immobilized in polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) after overnight incubation at 4ºC. The wells were washed twice with 250 µl of HH. Parallel, bacteria were grown overnight at 37ºC in MRS broth and the bacterial pellets from 1 ml fractions were obtained by centrifugation and washed with HH. Then, 10 µl of 10 mM carboxyfluorescein (Sigma) were added to the pellets and the bacterial suspensions were incubated for 20 min at 37ºC. Subsequently, the bacterial cells were washed 3 times with HH and, finally, resuspended in 1 ml of HH. Then, a suspension of 50 µl of the fluorescent-labelled bacteria (~ 5×10^7 cfu) was added to each well. After incubation for 1 h at 37ºC, the plates were washed twice with 250 µl

of HH to remove unattached cells, and incubated for 1 h at 60ºC in the presence of 50 µl of 1% sodium dodecyl sulphate (SDS)-0.1 M NaOH to release and lyse bound microorganisms. Fluorescence was measured in a fluorescence microplate reader (Tecan Austria GMBH, Salzburg, Austria). Adhesion was assessed as the percentage of the fluorescence retained in the wells after the washing steps when compared to that present in the labelled bacterial aliquots originally added to the wells. *L. reuteri* CR20 was used as a positive control (strong adherence to mucin) (Martín et al., 2009). The assays were performed in duplicate.

The potential of the lactobacilli strains to degrade gastric mucin (HGM; Sigma) *in vitro* was evaluated in duplicate following the procedure developed by Zhou et al. (2001).

IV.3.4. Antibiotic resistance/susceptibility

Minimum inhibitory concentrations (MICs) of the 16 antibiotics included in this study (gentamicin, kanamycin, streptomycin, neomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, penicillin, vancomycin, virginiamycin, linezolid, trimethoprim, ciprofloxacin and rifampin) were determined by a microdilution method using VetMIC plates for lactic acid bacteria (National Veterinary Institute of Sweden, Uppsala, Sweden), as described previously (Langa et al., 2012). The plates were incubated at 37°C for 48 h and MIC was defined as the lowest concentration at which no growth was observed. MICs values were compared with the microbiological cut-off parameters established by the European Food Safety Authority (EFSA, 2012) for *Lactobacillus* species.

IV.3.5. Prophage induction

In order to discard the presence of phages compromising strain viability, prophage induction was carried as described previously (Langa et al., 2012). Exponential cultures of the lactobacilli strains (A_{600}~0.4) were treated with 0.25, 0.5 (minimal inhibitory concentration; MIC), and 1 µg/ml mitomycin C (final concentration), and incubation was continued for up to 5 h. Aliquots of the supernatants were placed on lawns of presumably susceptible *L. salivarius* strains growing in soft MRS (0.75% agar) supplemented with 1% haemoglobin, 10 mM $CaCl_2$ and 10 mM $MgSO_4$, placed on top of plates with the same medium (1.5% agar). After incubation for 24 h, the generation (or not) of lysis plates was recorded.

IV.3.6. Production of biogenic amines

Initially, the ability to form biogenic amines (tyramine, histamine, putrescine and cadaverine) was assessed using the decarboxylase broth and the method described by Bover-Cid and Holzapfel (1999). The precursor amino acids (tyrosine, histidine, ornithine and lysine, respectively) were purchased from Sigma. The lactobacilli strains

were streaked onto the different decarboxylase medium plates and incubated for 4 days at 37ºC under aerobic and anaerobic conditions. A positive result was indicated by a change of the medium colour to purple in response to the pH shift caused by the production of the more alkaline biogenic amine from the amino acid initially included in the medium.

Parallel, the lactobacilli strains were grown for 24 h in MRS broth supplemented with 10 mM tyrosine (M17T), 13 mM of histidine (M17H) or 20 mM agmatine (M17A) for the detection of tyramine, histamine and putrescine production, respectively. The supernatants were filtered through a 0.2 μm pore diameter membrane, derivatyzed and analyzed by thin layer chromatography (TLC) following the conditions described by García-Moruno et al (2005).

In addition, the presence of the tyrosine decarboxylase gene (*tdcA*), histidine decarboxylase gene (*hdcA*) and agmatine deiminase cluster (*AgdDI*) was checked by specific PCR using methods described previously (Le Jeune et al., 1995; Lucas and Lonvaud-Funel, 2002; Ladero et al., 2012).

IV.4. *IN VIVO* ASSESSMENT OF THE SAFETY OF *L. salivarius* V4II-90 IN A RAT MODEL

Globally, *L. salivarius* V4II-90 was the strain that showed the best results as a candidate for future clinical trials. Subsequently, its potential acute and chronic oral toxicity was evaluated; in addition, the potential translocation of this strain to blood and some organs was also investigated.

IV.4.1. Acute and repeated doses (4-weeks) oral toxicity studies

Wistar male and female rats (Charles River Inc., Marget, Kent, UK) were acclimated for 7 days prior to study initiation with an evaluation of health status. The rats were individually housed in polycarbonate cages with sawdust bedding and maintained in environmentally controlled rooms (22 ± 2ºC and 50% ± 10% relative humidity) with a 12 h light–dark cycle (light from 08.00 to 20.00 h). Food (A03 rodent diet, Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France) and water were available ad libitum. The rats were 56-days old at the initiation of treatment.

Acute (limit test) and repeated dose (4 weeks) studies were conducted in accordance with the European Union guidelines. Both studies were undertaken in accordance with the ethics requirements and authorized by the Official Ethical Committee of the Complutense University.

In the acute (limit test) study, 24 rats (12 males, 12 females) were distributed into two groups of 6 males and 6 females each. After an overnight fast each rat received skim milk (500 μl) orally (control group or Group 1), or a single oral dose of 1×10^{10} cfu of

L. salivarius V4II-90 dissolved in 500 µl of skim milk (treated group or Group 2). Doses of the test and control articles were administered by gavage. Animals were checked for clinical signs and mortality twice a day (a.m. and p.m.). At the end of a 14 days observation period, the rats were weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied.

The repeated doses (4 weeks) (limit test) study was conducted in 48 rats (24 males, 24 females) divided in four groups of 6 males and 6 females each (control group or Group 3; treated group or Group 4; satellite control group or Group 5; and satellite treated group or Group 6). Rats received a daily dose of either skim milk (Groups 3 and 5) or 1×10^9 cfu of *L. salivarius* V4II-90 dissolved in 500 µl of skim milk (Groups 4 and 6) orally once a day over 4 weeks. Doses of the test and control articles were administered by gavage. Animals were dosed at approximately the same time each day (approximately 4–6 h into light cycle). Food but not water was withheld from 4 h before until 2 h after control and test article administration. Animals were checked for clinical signs and mortality twice a day (a.m. and p.m.). All rats of the Groups 3 and 4 were deprived of food for 18 h, weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied on Day 29. All animals of the satellite groups (Groups 5 and 6) were kept a further 14 days without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects. All rats of the Groups 5 and 6 were deprived of food for 18 h, weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied on Day 42.

IV.4.2. Animal observations

All animals were observed twice daily for general appearance, behaviour, sings of morbidity and mortality (once before treatment and once daily thereafter). Rats were observed for their general condition and the condition of the skin and fur, eyes, nose, oral cavity, abdomen and external genitalia, evaluated for respiration rate and palpated for masses. Behavioural parameters checked were abnormal movements (tremor, convulsion, muscular contractions), reactions to handling and behaviour in open field (excitability, responsiveness to touch and to sharp noise), changes in ordinary behaviour (changes in grooming, head shaking, gyration), abnormal behaviour (autophagia, backward motion) and aggression. Body weight, body weight gain and food and water consumption were measured daily and at the end of the observation periods the rats were examined by necropsy, and the weights of the organs recorded.

IV.4.3. Clinical test parameters

Blood samples for haematology and clinical chemistry evaluation were collected from the retro-orbital plexus from animals under light anaesthesia induced by CO_2 inhalation after 14 days observation period in the acute oral study and alter 4 weeks of treatment and 14 days of recovery for the repeated dose 4 weeks safety study. EDTA was used as an anticoagulant for haematology samples and sodium citrate was used as

an anticoagulant for clinical chemistry. Food was withheld for approximately 18 h before blood collection, and samples were collected early in the working day to reduce biological variation; water was provided *ad libitum*.

Clinical pathology parameters (haematological and clinical biochemistry) were evaluated. Most haematology variables were measured with a Coulter/CELL-DYN 3500 whole blood automated analyzer (Abbott, Chicago, IL). Blood cell smears were observed with an Olympus Microscopy BX41 (Olympus, Tokyo, Japan).

Clinical chemistry parameters were evaluated with a spectrophotometer Konelab PRIME 30 (Thermofisher Scientific Inc. Waltham, MA, USA) and special biochemistry parameters with a clinical chemistry analyzer AU640 (Olympus, Tokyo, Japan). Coagulation parameters were analyzed with a coagulation analyzer Coatron M1 (Teco Medical Instruments, GMBH, Neufahrn, Germany).

IV.4.4. Anatomical pathology

All rats were euthanized by CO_2 inhalation and necropsied. The necropsy included a macroscopic examination of the external surface of the body, all orifices, the cranial cavity, the brain and spinal cord, the nasal cavity and paranasal sinuses, and the thoracic, abdominal, and pelvic cavities and viscera. Descriptions of all macroscopic abnormalities were recorded. Samples of the following tissues and organs were collected from all animals at necropsy and fixed in neutral phosphate-buffered 4% formaldehyde solution: adrenal glands, brain, heart, ileum, jejunum, caecum, colon, duodenum, rectum, stomach, oesophagus, trachea, kidneys, liver, lungs, pancreas, spleen, skin, testicles with epididymes, ovaries with oviducts, bone marrow, thymus, thyroid and parathyroid glands, seminal vesicles, urinary bladder and uterus. The organ: body weight ratios were calculated. All organ and tissue samples for histopathological examination were processed, embedded in paraffin, cut at an approximate thickness of 2 to 4 mm, and stained with hematoxylin and eosin. Slides of all organs and tissues listed above were collected from all animals of the control and treated groups.

IV.4.5. Isolation of *L. salivarius* V4II-90 from feces and vaginal swabs samples

Once the rats were euthanized a sample of colon content and a vaginal swab was collected from each animal. These samples were diluted and inoculated onto MRS-C agar plates. Isolates identified as *L. salivarius* by MALDI-TOF were submitted to pulsed-field gel electrophoresis (PFGE) genotyping (Arroyo et al., 2010), and their profiles were compared with that of *L. salivarius* V4II-90.

IV.4.6. Bacterial translocation

Bacterial translocation was analysed in blood, liver and spleen. blood (50 µl) were cultured in de Man, Rogosa, Sharpe (MRS) agar medium and incubated at 37ºC during

48 h anaerobically. Tissue samples were homogenized in buffered peptone water (1 g/ml) and 100 µl of the resulting homogenates were cultured on MRS agar as previously mentioned. After 48 h, the plates were checked for the presence of lactobacilli. Positive growth on MRS agar plates was defined by the presence of even a single colony.

IV.4.7. Total liver glutathione (GSH) concentration

A portion of 100 mg of liver from each mouse were homogenized in a 7.5% trichloroacetic acid solution and homogenates were centrifuged at 3,000 × *g* for 10 min at 4ºC. Total glutathione concentration was measured in the supernatants using a colorimetric commercial kit (OxisResearch, Portland, OR). Briefly, 40 µl of the homogenates or the standards were added to each well of a microtiter plate, together with 40 ml of a reducing agent (tris[2-carboxyethyl] phosphine in HCl), 40 ml of a chromogen (1-methyl-3-chloro-7-trifluoromethylquinolinium methylsulfate in HCl) and 40 ml of color developer (NaOH). After an incubation at room temperature and in the dark for 30 min, optical density was measured at 415 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Hercules CA).

IV.4.8. Statistical analysis (for toxicity studies)

All data are expressed as means ± standard error of the mean (SEM) of the determinations. Differences between control and treated groups were evaluated with a one-way analysis of variance (ANOVA) followed by Dunnett's test, and differences were considered significant at $P < 0.05$.

IV.5. DESIGN OF A PCR ASSAY FOR SPECIFIC AND SENSITIVE DETECTION OF *S. agalactiae* IN BIOLOGICAL SAMPLES

IV.5.1. *In silico* design of GBS-specific primers

In order to design GBS species-specific primers, 16S rRNA gene sequences from various bacterial species (*S. agalactaie* and taxonomically-related species) available in the Genbank database were analyzed and compared using the programs Emma and Showalign, included in the free open source software analysis EMBOSS (The European Molecular Biology Open Software Suite).

IV.5.2. Conventional and Real-Time PCR assays: specificity and detection limit

All reactions were set up using the CFX Connect and CFX96 Touch™ Real-Time PCR Detection Systems (Bio-Rad Laboratories, Inc, Hercules, CA, USA). The reaction volume for conventional PCR was 25 µL, and 10 µL for real-time PCR amplification. Each run contained a negative control (deionized sterile water) and a positive control (100 or 25 ng of DNA from S. *agalactiae* DSMZ 2134). In the real-time assay the

determination of a PCR positive result and the specificity of the reaction was based on the melting curve. The melting temperature (Tm), which is specific for each amplicon, depends on various factors including the amplicon length and the nucleotide sequence.

The specificity of the primers designed was tested using DNA obtained from a panel of 6 *S. agalactiae* strains, 10 strains of other streptococcal species, and 21 strains of species belonging to related genera. All strains were analyzed in duplicate.

To check the detection limit of this PCR approach, the DNA extracted from pure culture of *S. agalactiae* DSMZ 2134, obtained from the German Collection of Microorganism and Cell Cultures (DSMZ), was used to prepare 10-fold dilution series with a bacterial population ranging from 2 to 9 $\log_{10}$ cfu, as determined by plate counting.

IV.5.3. Efficiency

The DNA extracted from vagino-rectal swab samples provided by female volunteers was analyzed in order to evaluate the applicability of the conventional and real-time PCR assay to the detection of *S. agalactiae* colonization in women. Both PCR approaches were compared with a conventional culture approach and GBS identification by MALDI-TOF, as explained above. All samples were analyzed in duplicate.

In the real-time PCR assay the presence or absence of GBS DNA in a sample was defined using a cut-off Ct value equal to 38.64 (the mean value less twice the standard deviation) of the non-target species and non-template controls). DNA from *S. agalactiae* was considered present if a Ct value < 38.64 was obtained.

IV.6. EFFICACY OF *L. salivarius* V4II-90 TO ERADICATE GBS FROM THE INTESTINAL AND VAGINAL TRACTS OF PREGNANT WOMEN: CLINICAL TRIAL

IV.6.1. Design of the trial

A total of 87 pregnant women (69 rectal and/or vaginal GBS-positive women; 18 rectal and vaginal GBS-negative women at the start of the intervention), aged 25-36, participated in this study. All met the following criteria: a normal pregnancy and a healthy status. Women ingesting probiotic supplements or receiving antibiotic treatment in the previous 30 days were excluded. All volunteers gave written informed consent to the protocol (10/017-E), which had been approved by the Ethical Committee of Clinical Research of Hospital Clínico San Carlos Madrid (Spain).

Volunteers were distributed into 3 groups (1 probiotic and 2 placebo groups). All the volunteers in any of the probiotic groups (n = 55) were GBS-positive and consumed a daily sachet with ~50 mg of freeze-dried probiotic (~9 $\log_{10}$ cfu of *L. salivarius* V4II-

90). Probiotic subgroup (n = 25) started the intervention at pregnancy weeks 26, respectively. Placebo subgroup 1 (n=14) included GBS-positive women (pregnancy week ranging from 19 to 30) that were going to receive intrapartum antibiotic prophylaxis (IAP) because they had a previous baby that suffered a GBS sepsis. Placebo subgroup 2 (n=18) included GBS-negative women (pregnancy week ranging from 14 to 26). Women in both placebo subgroups received a daily sachet containing 50 mg of the excipient used to carry the probiotic strain. In all cases, the intervention lasted from the start of the intervention to week 38, when recto-vaginal GBS screening was performed not only in our laboratory but, also, in those of the hospitals in which the respective women were going to deliver their babies. Probiotic- and excipient-containing sachets were kept at 4ºC throughout the study. All volunteers were provided with diaries to record compliance with study product intake. Minimum compliance rate (% of the total treatment doses) was set at 86%.

IV.6.2. Collection and GBS analysis of the samples

Rectal and vaginal exudates samples were collected periodically during the trial. They were serially diluted and plated on Granada (Biomerieux; isolation of haemolytic GBS, which appear as orange colonies), and CHROMagar StrepB (CHROMagar; for isolation of haemolytic and non- haemolytic GBS, which appear as purple colonies) agar plates. Correct taxonomic assignment was confirmed by MALDI-TOF and latex agglutination analyses, as described previously.

Parallel, and to avoid sensitivity-related problems, two strategies were used: (a) DNA was extracted from the samples and submitted to the PCR assays described above; (b) a subset of samples was submitted to a GBS enrichment step in Todd-Hewitt broth (Oxoid). After 24 h at 37ºC, the broth cultures were spread on CHROMagar StrepB agar plates.

IV.6.3. Statistical analysis

Microbiological data were recorded as CFU/ml and transformed to logarithmic values before statistical analysis. Two-way ANOVA was used to investigate the effect of individual (woman) and sampling time on the semiquantitative *S. agalactiae* counts in vaginal swabs. Statistical significance was set at $P< 0.05$. Statgraphics Centurion XVI version 17.0.16 (Statpoint Technologies Inc, Warrenton, Virginia) was used to carry out statistical analyses.

V. RESULTS

V.1. ASSESSMENT OF THE VAGINAL MICROBIOTA OF GBS-POSITIVE AND GBS-NEGATIVE FERTILE WOMEN (NON-PREGNANT AND PREGNANT)

A total of 54 fertile women (30 non-pregnant women and 24 pregnant women), aged 25-35, participated in this study. All volunteers gave written informed consent to the protocol, which had been approved (protocol 10/017-E) by the Ethical Committee of Clinical Research of Hospital Clínico San Carlos Madrid (Spain). In relation to non-pregnant women, 4 vaginal exudates samples were collected within a menstrual cycle (days 0, 7, 14 and 21). Pregnant women provided a single simple in week 35-37 of pregnancy. All women claimed to be completely healthy.

In all cases, bacterial growth was detected when the samples were inoculated on MRS, (2.70-8.08 log10 cfu; mean 5.36 log10 cfu); CNA (3.00-7.92 log10 cfu; mean 5.13 log10 cfu) and GAR (2.70-8.10 log10 cfu; mean 5.24 log10 cfu) agar plates. Globally, similar bacterial groups grew in the three media (data not shown). Growth on MCK, SDC or Mycoplasma plates was only detected in a few percentage of samples (from 0% in Mycoplasma plates to ~40% in SDC plates).

S. agalactiae could be isolated from both non-pregnant (~25%) and pregnant (~20%) women. *Candida albicans* and other yeasts were isolated from, approximately, 7 and 36% of the non-pregnant and pregnant women, respectively. In both groups, *Lactobacillus* were the dominant genus since it was detected in 92-93% of the participating women.

In relation to the samples provided by non-pregnant women, a total of 433 isolates (including, at least, one representative of each colony and cell morphology) were submitted to taxonomical analyses. The genus *Lactobacillus* was the dominant one (28% of the total isolates), followed by *Staphylococcus*, *Enterococcus*, *Corynebacterium* and *Streptococcus* (Table 1). Among the isolates, the main species were *L. crispatus* (23%), *L. gasseri* (24%), *L. salivarius* (21%), *L. vaginalis* (12%), *L. plantarum* (13%), *L. coleohominis* (5%) and *L. jensenii* (2%).

From the samples provided by pregnant women, 120 isolates were submitted to taxonomical analyses. Again, the genus *Lactobacillus* was the dominant one (17% of the total isolates), followed by *Staphylococcus, Streptococcus, yeasts* and *Enterococcus,* (Table 1). Among the isolates, the main species were *L. gasseri* (41%), *L. casei* (19%), *L. salivarius* (16%), *L. fermentum* (8%), *L. vaginalis* (6%), *L. reuteri* (5%) and *L. jensenii* (5%).

Table 1. Main genera and species isolated from the vaginal samples provided by the women that participated in the study.

Non-pregnant women			**Pregnant women**		
Genus/groups	Percentage (%)	Main species	Genus/groups	Percentage (%)	Main species
Lactobacillus	28	*L. crispatus* (23%) *L. gasseri* (24%) *L. salivarius* (21%) *L. vaginalis* (12%) *L. plantarum* (13%) *L. coleohominis* (5%) *L. jensenii* (2%)	*Lactobacillus*	18	*L. gasseri* (41%) *L. casei* (19%) *L. salivarius* (16%) *L. fermentum* (8%) *L. vaginalis* (6%) *L. reuteri* (5%) *L. jensenii* (5%)
Staphylococcus	17	*S. epidermidis* *S. hominis* *S. aureus*	*Staphylococcus*	17	*S. epidermidis* *S. hominis* *S. aureus*
Enterococcus	11	*E. faecalis*	*Streptococcus*	10	*S. agalactiae* (60%)
Corynebacterium	7	*C. pseudogenitalium* *C. afermentans* *C. aurimucosum*	Yeasts	10	*C. albicans* *C. glabrata,* *C. parapsilosis*
Streptococcus	4	*S. agalactiae* (75%) *S. sanguinis* *S. anginosus*	*Enterococcus*	6	*E. faecalis*
Others	12	*Streptomyces albus* *Dermabacter hominis* *Propionibacterium avidum* *Aerococcus urinae* *Dermacoccus nishinomiyaensis* *Actinomyces neuii* *Roseomonas mucosa* *Facklamia spp.* *Arthrobacter cumminsii* *Brevibacterium sp* *Helcobacillus massiliensis* *E. coli* Yeasts (*C. albicans, C. glabrata, C. parapsilosis*)	Others	12	*Bifidobacterium* spp.(30%) *Corynebacterium* spp. (10%)
Non-identified	21		Non-identified	27	

Overall, the dominant bacterial genus was the genus *Lactobacillus*, present in 85.71% of the samples from both groups of women (Figure 8).

Other genera such as *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Corynebacterium* were isolated from the samples of both groups of women (Figure 8). In addition, 35.71% of pregnant women had yeast and *Gardnerella vaginalis* was isolated in 7.14%. Regarding samples from non-pregnant women, yeasts were detected in a lower percentage of samples (7.14%) and enterobacteria (7%).

The presence of GBS was detected in 22% of the women in the study. The percentage of GBS positive women in the group of pregnant women was lower than that of non-pregnant women (19% and 25%, respectively). On the other hand, throughout the menstrual cycle, *S. agalactiae* was isolated with a higher frequency in the menstrual phase, being detected in 20% of the samples of EGB-positive women, in comparison with the rest of the phases, postmenstrual (5 %), ovulation (15%) and premenstrual (10%). However, the presence of EGB in the different phases of the cycle was not constant, varying individually. Thus, while in only one woman could be detected throughout the cycle (V6), in others it was only detected in two phases of the cycle (V16 and V20) or, exclusively during the menstrual phase (V2 and V14) (Figure 9).

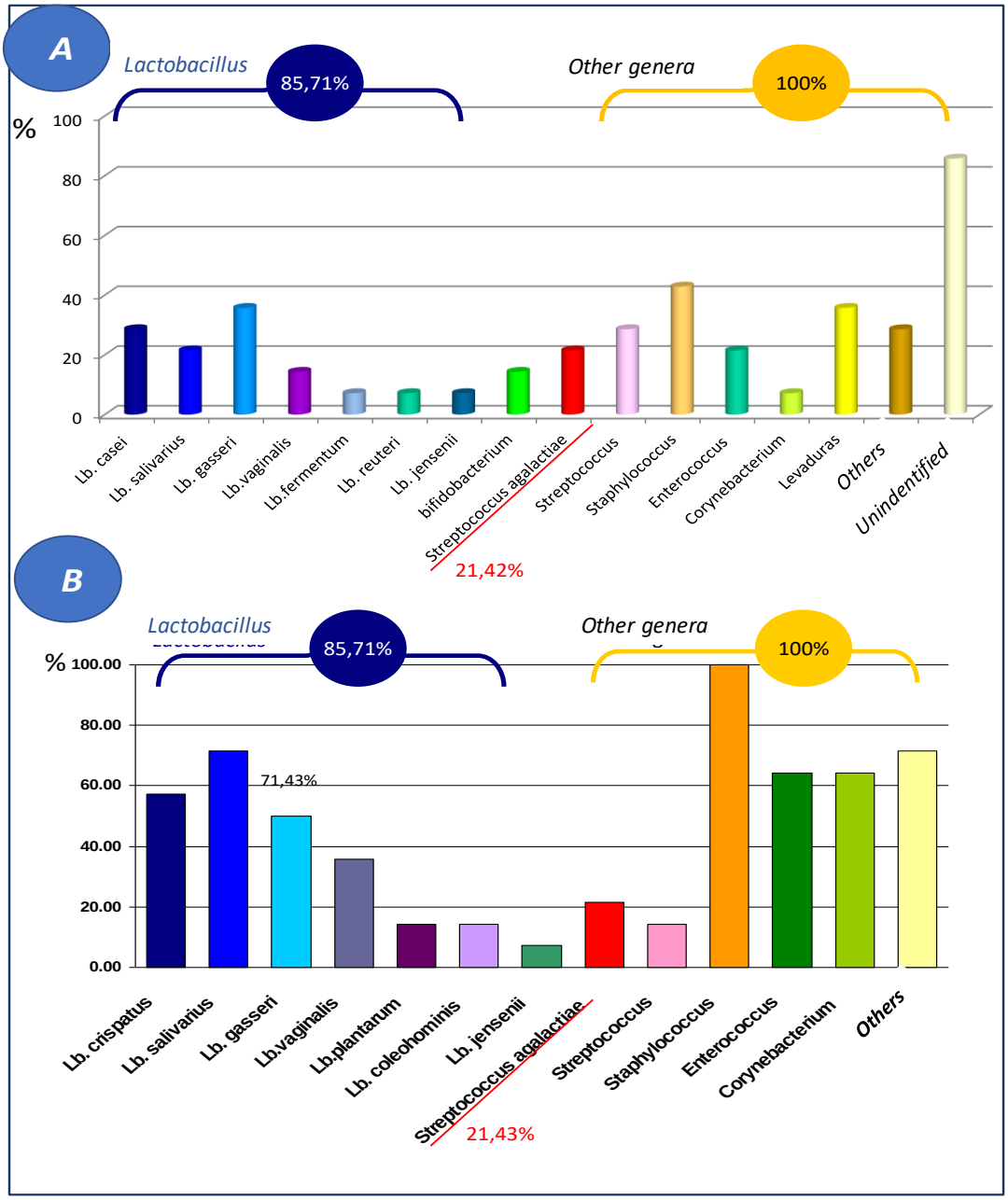


Figura 8. Frequency of detection of the different bacterial groups in pregnant women (A) and non-pregnant women (B).

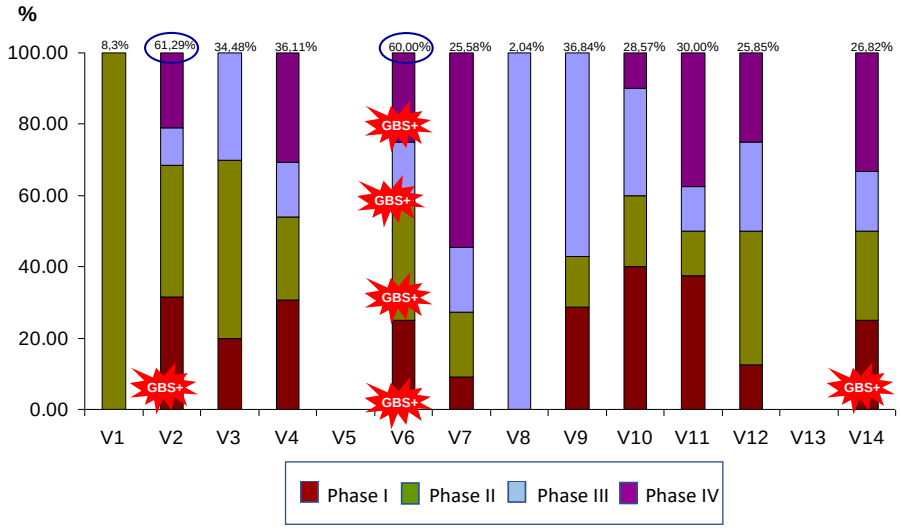


Figure 9. Distribution of lactobacilli throughout the menstrual cycle in each woman.

As stated above, the 89 different lactobacilli strains isolated in this study belonged to the following species*: Lactobacillus salivarius, Lactobacillus gasseri, Lactobacillus vaginalis, Lactobacillus jensenii, Lactobacillus crispatus, Lactobacillus plantarum, Lactobacillus colehominis, Lactobacillus casei, Lactobacillus reuteri* and *Lactobacillus fermentum*. Among them, a few were selected on the basis of the following criteria: (1) absence of *S. agalactiae, Gardnella vaginalis, Candida spp., Ureaplasma* spp. and *Mycoplasma spp* in the vaginal samples from which the lactobacilli were originally isolated; (2) Qualified Presumption of Safety (QPS) status (European Authority of Food Safety, EFSA) of the *Lactobacillus* species, since there was the purpose of using them as food supplements; and (3) ability of the strain to grew rapidly in MRS broth under aerobic conditions (≥1 ×106 cfu/mL after 16 h at 37ºC). Only 10 strains (V3III-1, V4II-90, V7II-1, V7II-62, V7IV-1, V7IV 60, V8III-62, V11I-60, V11III-60 y V11IV-60) met all the criteria and, interestingly, all of them belonged to the same species *(Lactobacillus salivarius*). These strains were those selected for further characterization.

V.2. ISOLATION AND CHARACTERIZACION OF THE SAFETY AND EFFICACY AGAINST GBS OF LACTOBACILLI ISOLATED IN THE PREVIOUS PHASE

V.2.1. Antimicrobial activity and production of potential antimicrobial compounds

Initially, the antimicrobial activity of the 10 selected lactobacilli against the *S. agalactiae* strains was determined by an overlay method. Clear inhibition zones (ranging from 2 to 20 mm) were observed around the lactobacilli streaks.

In relation to the antimicrobials compounds that may be responsible for such activity, the concentration of L- and D-lactic acid and the pH of the supernatants obtained from MRS cultures of the lactobacilli are shown in Table 2. The global concentration of L-lactic acid was similar (~10 mg/mL) in all the supernatants. In contrast, D-lactic acid was not detected in the supernatants of the tested strains. In addition, all the strains acidified the MRS-broth medium to a final pH of ~4 after 16 h of incubation; among them, *L. salivarius* V7IV-1 showed the highest acidifying capacity (final pH of 3.8).

No bacteriocin-like activity could be detected against the tested *S. agalactiae* strains. Two strains (*L. salivarius* V4II-90 and V7IV-1) were able to produce hydrogen peroxide (7.29 μg/mL ± 0.69 and 7.46 μg/mL ± 0.58, respectively) (Table 2).

Table 2. pH and concentrations of L- and D-lactic acid (mg/mL; mean ± SD), and hydrogen peroxide (μg/mL; mean ± SD) in the supernatants obtained from MRS cultures of the lactobacilli (n=4).

Strain	pH	L -lactic acid	D-lactic acid	Hydrogen peroxide
L. salivarius V3III-1	4.00	9.66 ± 0.57	Nd	Nd
L. salivarius V4II-90	4.01	10.03 ± 0.60	Nd	7.29 ± 0.69
L. salivarius V7II-1	4.02	9.82 ± 0.69	Nd	Nd
L. salivarius V7II-62	4.01	9.76 ± 0.54	Nd	Nd
L. salivarius V7IV-1	3.85	10.47 ± 0.58	Nd	7.46 ± 0.58
L. salivarius V7IV-60	4.02	9.72 ± 0.63	Nd	Nd
L. salivarius V8III-62	4.04	9.91 ± 0.55	Nd	Nd
L. salivarius V11I-60	4.03	9.84 ± 0.43	Nd	Nd
L. salivarius V11III-60	4.07	9.61 ± 0.47	Nd	Nd
L. salivarius V11IV-60	4.03	10.02 ± 0.62	Nd	Nd
L. salivarius CECT 5713	3.93	10.26 ± 0.62	Nd	-

The initial pH value of MRS broth was 6.2. ND: not detectable.

V.2.2. Production of biogenic amines

The *L. salivarius* strains neither produced biogenic amines nor harboured the genes required for the biosynthesis of this type of compounds.

V.2.3. Co-aggregation between the lactobacilli and the *S. agalactiae* strains

The capacity of the lactobacilli strains to form large, well defined co-aggregates *with S. agalactiae* was strain-dependent. Strains V3III-1, V7IV-60 and V11IV-60 coaggregated with 5 *S. agalactiae* strains, strains V8III-62, V11I-60 and V11III-60 with 7, strain V7II-62 with 9 *S. agalactiae* strains, and strains V4II-90, V7II-1 and V7IV-1 with 10 *S. agalactiae* strains (Figure 10).

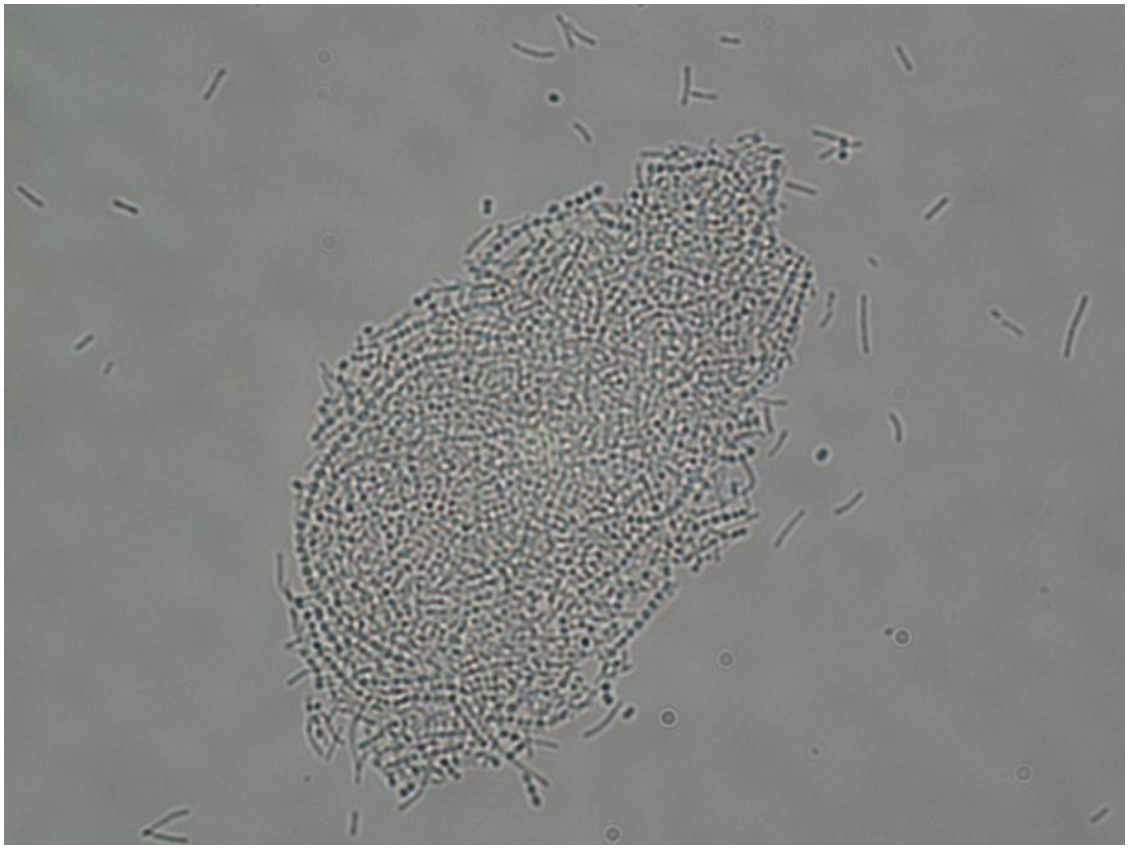


Figure 10. Strong co-aggregation between *L. salivarius* V4II-90 (rods) and a *S. agalactiae* strain (cocci chains).

V.2.4. Broth co-cultures of the lactobacilli and the *S. agalactiae* strains

The ability of the lactobacilli strains to interfere or inhibit the growth of four *S. agalactiae* strains was evaluated using MRS broth co-cultures. Co-cultures with *S. agalactiae* seemed not to affect the growth of any of the *L. salivarius* strains (Table 3). In contrast, most of *the L. salivarius* strains were able to interfere at a higher or lower degree with the growth of the different *S. agalactiae* strains included in this assay. Among them, *L. salivarius* V4II-90 showed the highest ability to inhibit the growth of *S. agalactiae* since the presence of two of the four *S. agalactiae* strains was not detectable in the co-cultures and the concentration of the other two showed a ~2.5 log10 decrease after an incubation period of only 6 h at 37ºC (Table 3). Interestingly, no viable streptococci could be detected when the in the co-cultures were incubated for 24 h (Table 3).

Table 3. Bacterial counts of the *S. agalactiae* strains when co-cultured with the *L. salivarius* strains in MRS broth for 0, 6 and 24 h at 37ºC.

***L. salivarius* (strain)**	***S. agalactiae* (strain)**	**0 h**	**6 h**	**24 h**
V3III-1	RC5	7.10	6.44	Nd
	RC6	7.24	7.04	Nd
	V2I-80	7.10	7.04	Nd
	V14I-63	7.27	7.10	Nd
V4II-90	RC5	7.04	4.48	Nd
	RC6	7.23	Nd	Nd
	V2I-80	7.10	4.70	Nd
	V14I-63	7.34	Nd	Nd
V7II-1	RC5	7.15	7.27	Nd
	RC6	7.15	6.70	Nd
	V2I-80	7.04	7.10	Nd
	V14I-63	7.35	5.65	Nd
V7II-62	RC5	7.24	7.04	Nd
	RC6	6.98	7.49	Nd
	V2I-80	7.35	7.92	Nd
	V14I-63	7.10	6.93	Nd
V7IV-1	RC5	7.32	7.58	Nd
	RC6	7.34	6.90	Nd
	V2I-80	7.15	7.38	Nd
	V14I-63	7.23	6.04	Nd
V7IV-60	RC5	7.24	7.32	Nd
	RC6	7.32	8.06	Nd
	V2I-80	7.04	7.15	Nd
	V14I-63	7.35	8.34	Nd
V8III-62	RC5	7.15	7.90	Nd
	RC6	7.34	7.23	Nd
	V2I-80	7.24	6.90	Nd
	V14I-63	7.20	8.77	Nd
V11I-60	RC5	7.31	7.44	Nd
	RC6	7.01	6.94	Nd
	V2I-80	7.23	7.07	Nd
	V14I-63	6.93	6.60	Nd
V11 III-60	RC5	7.27	6.44	Nd
	RC6	6.95	6.88	Nd
	V2I-80	7.28	6.52	Nd
	V14I-63	7.37	6.85	Nd
V11IV-60	RC5	7.26	6.74	Nd
	RC6	7.42	6.60	Nd
	V2I-80	7.10	6.60	Nd
	V14I-63	7.06	5.32	Nd
Control cultures (no *L. salivarius* strain)	RC5	7.20	9.32	9.34
	RC6	7.31	9.20	9.27
	V2I-80	7.04	9.15	9.23
	V14I-63	7.10	9.02	9.15

V.3. *IN VITRO* ASSESSMENT OF OTHER PROBIOTIC PROPERTIES OR PRERREQUISITES OF THE LACTOBACILLI STRAINS

V.3.1. Survival after transit through an *in vitro* gastrointestinal model

The viability of the strains after exposition to conditions simulating those found in the gastrointestinal tract varied from ~64% (*L. reuteri* CR20, *L. salivarius* V4II-90) to 30% (*L. salivarius* V3III-1) (Table 4).

Table 4. Percentage (%) of initial lactobacilli (10^9 cfu/mL) that survived to conditions simulating those of the gastrointestinal tract.

	Gastric-emptying fraction[a]				
Strain	**20 min**	**40 min**	**60 min**	**80 min**	**% Total**
L. salivarius V3III-1	9.4 ± 0.4	12.1 ± 0.5	5.0 ± 0.3	3.7 ± 0.1	30.2
L. salivarius V4II-90	16.9 ± 0.6	21.3 ± 1.7	16.5 ± 0.9	9.3 ± 0.5	64.3
L. salivarius V7II-1	14.8 ± 0.4	23.7 ± 2.4	14.3 ± 1.4	7.0 ± 0.4	59.8
L. salivarius V7II-62	14.3 ± 2.0	12.0 ± 1.3	11.5 ± 0.7	12.7 ± 1.5	50.5
L. salivarius V7IV-1	13.6 ± 0.8	13.5 ± 1.2	11.9 ± 1.3	9.1 ± 1.4	48.1
L. salivarius V7IV-60	12.8 ± 1.9	16.3 ± 1.5	10.7 ± 1.2	13.5 ± 1.6	53.3
L. salivarius V8III-62	7.5 ± 0.6	12.4 ± 1.0	10.8 ± 1.2	10.6 ± 1.4	41.3
L. salivarius V11I-60	8.2 ± 0.7	11.7 ± 1.4	11.3 ± 1.1	9.6 ± 1.3	40.8
L. salivarius V11III-60	7.9 ± 0.8	12.0 ± 1.2	10.2 ± 1.3	11.0 ± 1.6	41.1
L. salivarius V11IV-60	8.5 ± 0.7	12.7 ± 1.3	10.9 ± 0.9	10.2 ± 1.1	42.3
L. salivarius CELA2	15.4 ± 1.6	25.8 ± 2.9	17.0 ± 2.2	6.2 ± 0.4	64.4

V.3.2. Adherence assays to intestinal and vaginal epithelial cells

In this study, the lactobacilli strains tested were strongly adhesive to both Caco-2 and HT-29 cells, with the exception of the negative control strain (*L. casei* imunitass) which showed a low adhesive potential (Table 5). In addition, all showed adhesion to vaginal epithelial cells. Among the *L. salivarius* strains, *L. salivarius* V4II-90 globally displayed the highest ability to adhere to both intestinal and vaginal epithelial cells (Table 5).

V.3.3. Adherence to and/or degradation of mucin

The lactobacilli strains tested showed a variable ability to adhere to porcine mucin (Table 6). *Lb. reuteri* CR20 (positive control strain) was the strain that showed the highest adherence ability followed by *L. salivarius* V4II-90 and *L. salivarius* V7IV-1 (Table 6). None of the strains were able to degrade gastric mucin in vitro.

V.3.4. Antibiotic susceptibility

The MIC values of the lactobacilli strains for 16 antibiotics assayed are shown in Table 7. All the strains were sensitive to most of the antibiotics tested, including those considered clinically relevant antibiotics such as, gentamycin, tetracycline, clindamycin, chloramphenicol, and ampicillin, showing MICs equal to, or lower than, the breakpoints defined by EFSA (EFSA, 2012). All the strains were resistant to vancomycin and kanamycin, which is an intrinsic property of the *L. salivarius* at the species level.

Table 5. Ability of the lactobacilli to adhere to HT-29, Caco-2 and vaginal epithelial cells.

Strain	HT-29	Caco-2	Vaginal cells
L. salivarius V3III-1	877.3 ± 303.2	259.1 ± 67.1	+
L. salivarius V4II-90	905.2 ± 297.0	345.1 ± 72.8	+++
L. salivarius V7II-1	900.5 ± 336.2	297.8 ± 84.5	++
L. salivarius V7II-62	911.7 ± 250.9	321.5 ± 80.2	++
L. salivarius V7IV-1	884.0 ± 226.3	252.3 ± 67.1	++
L. salivarius V7IV-60	799.7 ± 210.1	255.9 ± 60.3	++
L. salivarius V8III-62	623.4 ± 200.2	108.7 ± 24.3	+
L. salivarius V11I-60	593.2 ± 191.5	121.6 ± 22.0	+
L. salivarius V11III-60	612.4 ± 188.2	153.2 ± 26.7	+
L. salivarius V11IV-60	601.6 ± 172.0	159.5 ± 23.4	+
L. rhamnosus GG	912.4 ± 345.0	371.5 ± 67.8	Nd
L. casei imunitass	127.4 ± 20.9	16.7 ± 7.3	Nd

The adherent lactobacilli in 20 random microscopic fields were counted for each test (n=4). Nd, not determined.

Table 6. Ability of the lactobacilli to adhere to porcine mucin.

Strain	Adhesion[a]
L. salivarius V3III-1	9.3 ± 2.0
L. salivarius V4II-90	10.9 ± 1.8
L. salivarius V7II-1	8.9 ± 1.9
L. salivarius V7II-62	9.0 ± 1.6
L. salivarius V7IV-1	8.5 ± 1.2
L. salivarius V7IV-60	9.6 ± 1.7
L. salivarius V8III-62	3.3 ± 0.7
L. salivarius V11I-60	2.9 ± 0.8
L. salivarius V11III-60	2.4 ± 1.0
L. salivarius V11IV-60	3.4 ± 0.8
L. reuteri CR20	11.8 ± 1.9

[a]Values expressed as the percentage of the fluorescence retained in the wells after the washing steps of the assay.

Table 7. Minimal inhibitory concentration (MIC, mg /ml) values of 16 antibiotics[a] to the *L. salivarius* strains.

	Antibiotic															
Strain	GEN	KAN	STP	NEO	TET	ERY	CLI	CHL	AMP	PEN	VAN	VIR	LIN	TRM	CIP	RIF
V3III-1	4	64	32	8	2	0.12	0.5	2	0.5	0.12	>128	0.5	0.5	0.5	2	0.5
V4II-90	4	256	32	8	2	0.12	0.5	2	0.5	0.12	>128	0.5	1	0.25	4	1
V7II-1	4	128	32	4	2	0.12	0.5	4	0.5	0.12	>128	0.5	0.5	0.5	2	0.25
V7II-62	2	128	32	8	2	0.25	0.5	2	0.5	0.25	>128	0.25	1	0.25	2	0.5
V7IV-1	8	256	32	4	2	0.12	0.5	2	0.5	0.25	>128	0.5	1	0.5	2	0.5
V7IV-60	8	128	32	8	2	0.12	0.4	4	0.5	0.25	>128	0.5	1	0.5	2	0.5
V8III-62	8	128	32	2	2	0.25	0.5	4	0.5	0.25	>128	1	1	0.5	2	0.5
V11I-60	4	128	32	8	2	0.12	0.5	2	0.5	0.25	>128	1	1	0.5	2	0.5
V11III-60	8	256	32	4	2	0.12	0.5	2	0.5	0.25	>128	0.5	1	0.5	2	0.5
V11IV-60	4	128	32	8	2	0.12	0.5	2	0.5	0.25	>128	1	1	0.5	2	0.5
Breakpoint[b]	16	R	64	nr	8	1	1	4	4	nr	nr (R)	nr	nr	nr	nr	nr

[a]Abbreviations: GEN, gentamycin; KAN, kanamycin; STP, streptomycin; NEO, neomycin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; AMP, ampicillin; PEN, penicillin; VAN, vancomycin; VIR, virginiamycin; LIN, linezolid; TRM, trimethoprim; CIP, ciprofloxacin; RIF, rifampicin; nr, not required by EFSA. R, the species *L. salivarius* is intrinsically resistant.
[b]Breakpoint: microbiological breakpoints (mg /ml) that categorise *Lactobacillus salivarius* as resistant (microbiological breakpoints are defined as the MIC values that clearly deviate from those displayed by the normal susceptible populations; EFSA, 2012).

V.3.5. Prophage induction

None of the supernatants obtained from the *L. salivarius* strains generated phage-related inhibition halos or plaques of lysis on lawns of any of indicator strains tested in this study.

IV.4. *IN VIVO* ASSESSMENT OF THE SAFETY OF *Lactobacillus salivarius* V4II-90 IN A RAT MODEL

V.4.1. Acute oral toxicity in rats

No abnormal clinical signs, behavioural changes, body weight changes, macroscopic findings, or organ weight changes were observed. All animals survived the 2-week observation period. There were no statistical differences in body weights among groups. Similarly, no statistically significant differences in body weight gain, food and water consumption were noted. Body weight, daily body weight gain, food and water consumption thus were unaffected by the treatment (single oral dose of 1×10^{10} cfu of *L. salivarius* V4II-90).

The haematological and clinical chemistry parameters assessed 2 weeks after administration of the strain as a single oral dose of 1×10^{10} cfu were not significantly different compared with those of controls (Tables 8 and 9). No treatment-related changes were noted.

There were no statistical differences in organ weight or tissue: body weight ratios related to the test strain (data not shown). The *L. salivarius* V4II-90 preparation was not associated with any incidence of macroscopic and microscopic changes. No treatment-related histopathological changes were observed 2 weeks after administration of the

strain as a single oral dose of 1×10^{10} cfu. Therefore, *L. salivarius* V4II-90 has a low order of acute toxicity and the oral lethal dose (LD50) for male and female rats is higher than 1×10^{10} cfu.

Table 8. Haematological parameters in rats after the 2-week observation period following a single oral dose of *L. salivarius* V4II-90 at 10^{10} cfu

	Group 1 (control)		Group 2 (probiotic)	
Parameters[a]	Female	Male	Female	Male
RBC (x10^6/ml)	8.33 ± 0.31	8.34 ± 0.25	8.34 ± 0.55	8.35 ± 0.47
Haemoglobin (g/dl)	14.69 ± 0.49	14.87 ± 0.47	14.73 ± 0.54	14.85 ± 0.61
Haematocrit (%)	47.47 ± 0.60	47.64 ± 0.59	47.43 ± 0.69	47.69 ± 0.64
MCV (fl)	57.33 ± 1.06	58.43 ± 0.92	57.03 ± 0.87	58.49 ± 1.29
MCH (pg)	17.65 ± 0.57	17.75 ± 0.64	17.70 ± 0.55	17.74 ± 0.67
MCHC (g/dl)	31.63 ± 0.54	31.82 ± 0.43	31.59 ± 0.49	31.84 ± 0.43
RDW (%)	15.70 ± 0.42	15.78 ± 0.44	15.65 ± 0.56	15.82 ± 0.50
WBC (×103/ml)	9.64 ± 0.72	9.90 ± 0.84	9.58 ± 0.65	9.93 ± 0.83
Banded neutrophils (×10^3/ml)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Neutrophils (×10^3/ml)	1.47 ± 0.20	1.49 ± 0.15	1.46 ± 0.17	1.50 ± 0.26
Eosinophils (×10^3/ml)	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
Lymphocytes (×10^3/ml)	7.02 ± 0.62	9.01 ± 0.52	7.11 ± 0.56	9.03 ± 0.60
Monocytes (×10^3/ml)	0.32 ± 0.07	0.35 ± 0.08	0.29 ± 0.07	0.34 ± 0.07
Basophils (×10^3/ml)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelets (×10^3/ml)	713.10 ± 35.23	729.69 ± 29.24	716.76 ± 38.12	731.61 ± 33.33
MPV (fl)	6.31 ± 0.14	6.80 ± 0.15	6.33 ± 0.13	6.80 ± 0.11

[a]Data are expressed as mean ± SEM (n = 6 animals/sex group).

Table 9. Clinical chemistry parameters in rats after the 2-week observation period following a single oral dose of *L. salivarius* V4II-90 at 10^{10} cfu

	Group 1 (control)		Group 2 (probiotic)	
Parameters[a]	Female	Male	Female	Male
Glucose (mg/dl)	119.02 ± 4.27	122.21 ± 4.42	119.92 ± 4.28	121.47 ± 5.32
Urea nitrogen (mg/dl)	48.67 ± 4.09	44.32 ± 3.76	49.33 ± 3.55	44.07 ± 4.53
Creatinine (mg/dl)	0.60 ± 0.07	0.53 ± 0.09	0.59 ± 0.07	0.52 ± 0.06
Total protein (g/dl)	7.53 ± 0.42	6.84 ± 0.34	7.51 ± 0.43	6.86 ± 0.41
Total bilirubin (mg/dl)	0.17 ± 0.03	0.18 ± 0.05	0.17 ± 0.04	0.18 ± 0.04
Calcium (mg/dl)	11.92 ± 0.56	11.84 ± 0.46	11.88 ± 0.44	11.85 ± 0.35
Sodium (mEq/l)	145.32 ± 6.04	148.40 ± 7.42	144.10 ± 6.52	149.24 ± 7.07
Potassium (mEq/l)	5.88 ± 0.47	6.42 ± 0.62	5.82 ± 0.51	6.45 ± 0.57
ASAT (u/l)	150.21 ± 7.84	157.44 ± 8.56	151.74 ± 8.14	157.58 ± 7.69
ALAT (u/l)	62.75 ± 5.82	70.19 ± 6.21	61.96 ± 5.65	70.72 ± 6.13
Alkaline phosphatase (u/l)	277.31 ± 35.25	823.85 ± 39.69	270.88 ± 37.46	820.36 ± 40.21
Triglyceride (mg/dl)	107.14 ± 8.46	128.10 ± 8.31	104.11 ± 9.31	126.56 ± 9.24
Cholesterol (mg/dl)	66.21 ± 5.47	62.21 ± 5.69	66.05 ± 6.24	62.46 ± 5.92
HDL (mg/dl)	47.33 ± 3.72	42.08 ± 4.01	46.82 ± 4.05	42.31 ± 4.02
LDL (mg/dl)	7.67 ± 0.76	8.46 ± 0.82	7.65 ± 0.79	8.44 ± 0.69

[a]Data are expressed as mean ± SEM (n = 6 animals/sex group).

V.4.2. Repeated dose (4 weeks) oral toxicity in rats

No mortality was observed. No treatment-related changes in the general condition and external appearance were observed in male and female rats treated with 1×10^9 cfu of *L. salivarius* V4II-90 daily dose. The development of the animals during the experimental period corresponded to their species and age. There was no significant difference in body weight or body weight gain among groups treated with *L. salivarius* V4II-90 in comparison to the control groups at any time point of the experimental period. All *L. salivarius* V4II-90-treated groups consumed similar amounts of food and water (data not shown) to that of the corresponding control groups.

All haematology data were within normal limits and differences between groups were no observed (Table 10). Clinical chemistry data showed no treatment-related alterations at the end of 4-weeks treatment period (Table 11). Individual values and group mean values were within the physiologic ranges. After 14 days without treatment

to detect delayed occurrence of potential toxic effects, there were no treatment-related changes neither in hematological nor in clinical test parameters (Tables 10 and 11; satellite control group or Group 5 and satellite treated group or Group 6).

Table 10. Haematological parameters in rats after repeated (4 weeks) oral doses of *L. salivarius* V4II-90 at 10^9 cfu.

	Group 3 (control)		Group 4 (probiotic)		Group 5 (satellite control)		Group 6 (satellite probiotic)	
Parameters[a]	Female	Male	Female	Male	Female	Male	Female	Male
RBC (x106/ml)	8.75 ± 0.17	8.84 ± 0.16	8.71 ± 0.17	8.85 ± 0.14	8.73 ± 0.18	8.84 ± 0.17	8.74 ± 0.14	8.86 ± 0.19
Haemoglobin (g/dl)	16.62 ± 0.43	16.75 ± 0.63	16.42 ± 0.62	16.81 ± 0.57	16.53 ± 0.62	16.80 ± 0.61	16.47 ± 0.62	16.82 ± 0.61
Haematocrit (%)	45.96 ± 2.24	47.07 ± 3.31	45.87 ± 2.66	47.05 ± 2.54	46.11 ± 3.92	46.97 ± 3.73	46.24 ± 2.88	47.16 ± 2.79
MCV (fl)	51.74 ± 0.99	52.96 ± 0.84	51.69 ± 0.77	52.87 ± 0.94	51.60 ± 0.89	53.02 ± 0.97	52.73 ± 1.37	52.86 ± 0.97
MCH (pg)	18.24 ± 0.55	18.79 ± 0.51	18.26 ± 0.53	18.77 ± 0.64	18.35 ± 0.59	18.74 ± 0.67	18.24 ± 0.55	18.76 ± 0.65
MCHC (g/dl)	34.89 ± 0.94	35.44 ± 0.85	34.99 ± 0.79	35.46 ± 0.87	35.05 ± 0.99	35.19 ± 0.88	34.99 ± 0.81	35.45 ± 1.05
RDW (%)	18.22 ± 0.65	17.96 ± 0.54	17.93 ± 0.68	18.04 ± 0.49	18.07 ± 0.57	17.96 ± 0.51	18.06 ± 0.65	18.06 ± 0.77
WBC ($\times 10^3$/ll)	7.68 ± 0.69	9.96 ± 0.66	7.65 ± 0.76	10.13 ± 0.57	7.69 ± 0.59	9.98 ± 0.77	7.65 ± 0.49	9.97 ± 0.79
Banded neutrophils ($\times 10^3$/ml)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Neutrophils ($\times 10^3$/ml)	1.06 ± 0.14	1.48 ± 0.14	1.06 ± 0.13	1.48 ± 0.17	1.04 ± 0.12	1.46 ± 0.15	1.02 ± 0.16	1.45 ± 0.13
Eosinophils ($\times 10^3$/ml)	0.14 ± 0.03	0.18 ± 0.03	0.12 ± 0.03	0.16 ± 0.03	0.12 ± 0.02	0.17 ± 0.03	0.11 ± 0.03	0.15 ± 0.02
Lymphocytes ($\times 10^3$/ml)	7.77 ± 0.54	7.94 ± 0.55	7.83 ± 0.61	8.03 ± 0.65	7.94 ± 0.63	7.98 ± 0.68	7.69 ± 0.56	8.01 ± 0.62
Monocytes ($\times 10^3$/ml)	0.25 ± 0.06	0.41 ± 0.05	0.25 ± 0.04	0.43 ± 0.05	0.26 ± 0.03	0.43 ± 0.04	0.24 ± 0.03	0.42 ± 0.06
Basophils ($\times 10^3$/ml)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelets ($\times 10^3$/ml)	763.1 ± 42.1	783.7 ± 37.7	760.6 ± 42.3	781.9 ± 52.1	762.2 ± 37.3	786.0 ± 34.0	764.6 ± 37.7	788.0 ± 35.6
MPV (fl)	8.64 ± 0.27	8.74 ± 0.24	8.66 ± 0.36	8.72 ± 0.28	8.64 ± 0.29	8.72 ± 0.33	8.69 ± 0.35	8.75 ± 0.29
Prothrombin time (s)	17.64 ± 0.36	17.97 ± 0.38	17.74 ± 0.28	18.04 ± 0.35	17.74 ± 0.33	18.08 ± 0.35	17.53 ± 0.36	18.05 ± 0.36
Thromboplastin partial time (s)	30.28 ± 1.92	25.02 ± 1.85	31.04 ± 1.92	24.96 ± 1.78	29.57 ± 1.94	25.34 ± 1.77	29.99 ± 1.84	24.94 ± 1.96
Fibrinogen (mg/dl)	235.4 ± 13.2	305.2 ± 12.1	229.5 ± 13.4	297.6 ± 12.9	244.8 ± 16.4	300.6 ± 24.7	242.6 ± 15.6	311.7 ± 19.7

[a]Data are expressed as mean ± SEM (n = 6 animals/sex group).

Table 11. Biochemical parameters in rats after repeated (4 weeks) oral doses of *L. salivarius* V4II-90 at 10^9 cfu.

	Group 3 (control)		Group 4 (probiotic)		Group 5 (satellite control)		Group 6 (satellite probiotic)	
Parameters[a]	Female	Male	Female	Male	Female	Male	Female	Male
Glucose (mg/dl)	105.3 ± 4.4	121.3 ± 4.6	103.6 ± 3.8	120.3 ± 3.7	106.4 ± 4.9	119.5 ± 5.1	105.7 ± 4.9	121.2 ± 5.6
Urea nitrogen (mg/dl)	42.18 ± 1.93	39.38 ± 1.73	41.75 ± 2.13	38.82 ± 2.45	41.26 ± 2.60	41.65 ± 2.91	42.06 ± 2.89	39.75 ± 2.97
Creatinine (mg/dl)	0.67 ± 0.07	0.64 ± 0.05	0.65 ± 0.04	0.63 ± 0.05	0.67 ± 0.06	0.65 ± 0.06	0.68 ± 0.06	0.65 ± 0.04
Albumine (g/dl)	3.81 ± 0.27	3.61 ± 0.25	3.78 ± 0.29	3.58 ± 0.36	3.84 ± 0.29	3.53 ± 0.34	3.78 ± 0.27	3.57 ± 0.27
Total protein (g/dl)	6.75 ± 0.26	6.46 ± 0.28	6.68 ± 0.32	6.52 ± 0.26	6.79 ± 0.29	6.55 ± 0.25	6.72 ± 0.30	6.56 ± 0.19
Total bilirubin (mg/dl)	0.19 ± 0.04	0.18 ± 0.04	0.19 ± 0.03	0.17 ± 0.03	0.19 ± 0.05	0.19 ± 0.03	0.20 ± 0.03	0.21 ± 0.04
Calcium (mg/dl)	12.25 ± 0.33	11.84 ± 0.39	12.46 ± 0.38	12.09 ± 0.39	12.39 ± 0.39	12.04 ± 0.39	12.34 ± 0.38	12.02 ± 0.43
Sodium (mEq/l)	138.9 ± 3.9	139.8 ± 3.3	137.6 ± 3.9	138.9 ± 3.9	139.2 ± 3.9	140.5 ± 3.7	140.5 ± 3.5	140.4 ± 3.4
Potassium (mEq/l)	6.01 ± 0.36	6.13 ± 0.33	5.99 ± 0.29	6.03 ± 0.29	6.11 ± 0.32	5.98 ± 0.29	6.04 ± 0.29	5.99 ± 0.32
ASAT (u/l)	148.3 ± 6.2	141.4 ± 6.1	145.7 ± 7.5	140.9 ± 6.4	144.2 ± 6.4	142.9 ± 6.4	144.9 ± 7.1	141.3 ± 6.6
ALAT (u/l)	45.96 ± 2.04	43.25 ± 2.31	45.75 ± 2.12	42.94 ± 2.51	45.61 ± 2.67	43.52 ± 3.00	45.49± 2.81	43.65 ± 2.74
Alkaline phosphatase (u/l)	299.7 ± 20.1	477.3 ± 29.0	287.6± 27.4	469.5 ± 26.0	294.9 ± 24.3	472.1 ± 26.0	299.7 ± 37.5	474.0 ± 30.8
Triglyceride (mg/dl)	140.0 ± 11.5	177.8 ± 11.2	139.0 ± 12.2	173.0 ± 13.1	141.8 ± 12.9	175.0 ± 15.7	141.9 ± 15.7	174.8 ± 14.8
Cholesterol (mg/dl)	58.55 ± 2.52	65.77 ± 2.55	57.02 ± 2.96	64.06 ± 2.65	58.35 ± 3.53	65.45 ± 3.70	57.81 ± 3.85	64.95 ± 3.67
HDL (mg/dl)	39.53 ± 1.67	37.24 ± 1.55	38.88 ± 1.93	37.09 ± 1.99	39.07 ± 1.89	38.25 ± 3.01	38.76 ± 2.74	37.56 ± 2.86
LDL (mg/dl)	7.64 ± 0.97	8.03 ± 0.84	7.54 ± 0.85	8.17 ± 1.00	7.54 ± 1.16	8.14 ± 1.04	8.16 ± 0.94	8.05 ± 1.17
Lipoprotein A (mg/dl)	<2	<2	<2	<2	<2	<2	<2	<2

[a]Data are expressed as mean ± SEM (n = 6 animals/sex group).

The necropsy performed on day 29 after the last dose of *L. salivarius* V4II-90 (Group 4) and on day 42 after 14 days without any treatment (Group 6) did not reveal any gross pathological changes or any differences in organ weights in comparison to the corresponding control groups. Mean organ weights and rate body weight: organ are presented in Table 12. After 4-weeks of treatment, there were no treatment-related histopathological findings in the organs examined neither in male nor in female rats (data not shown). There were also no treatment-related histopathological findings in the satellite treated group (Group 6) (data not shown).

The no-observed-adverse-effect level in this repeated dose (4 weeks) oral toxicity study was the dose tested, i.e. 1×10^9 cfu of *L. salivarius* V4II-90.

Table 12. Mean organ weights and rate body weight/organ in rats after repeated (4 weeks) oral doses of *L. salivarius* V4II-90 at 10^9 cfu.

	Group 3 (control)		Group 4 (probiotic)		Group 5 (satellite control)		Group 6 (satellite probiotic)	
Parameters[a]	Female	Male	Female	Male	Female	Male	Female	Male
Body weight (g)	238.8 ± 7.6	327.2 ± 9.5	237.0 ± 8.2	333.4 ± 8.2	243.6 ± 7.6	383.5 ± 7.7	245.4 ± 6.8	378.4 ± 8.1
Increase body weight (g)	46.9 ± 3.1	117.6 ± 7.5	46.0 ± 5.7	120.7 ± 5.3	49.7 ± 4.7	173.3 ± 5.7	50.3 ± 3.7	169.8 ± 8.2
Brain weight (g)	1.80 ± 0.05	1.89 ± 0.06	1.82 ± 0.07	1.85 ± 0.06	1.77 ± 0.07	1.91 ± 0.06	1.77 ± 0.05	1.87 ± 0.07
Rate body weight/brain (%)	0.75 ± 0.04	0.57 ± 0.04	0.76 ± 0.05	0.55 ± 0.04	0.72 ± 0.06	0.49 ± 0.04	0.72 ± 0.05	0.49 ± 0.04
Thymus weight (g)	0.42 ± 0.03	0.68 ± 0.06	0.44 ± 0.04	0.64 ± 0.07	0.43 ± 0.04	0.55 ± 0.04	0.43 ± 0.05	0.58 ± 0.06
Rate body weight/thymus (%)	0.16 ± 0.03	0.20 ± 0.04	0.17 ± 0.04	0.18 ± 0.04	0.17 ± 0.05	0.15 ± 0.06	0.17 ± 0.04	0.15 ± 0.06
Heart weight (g)	0.64 ± 0.06	0.94 ± 0.08	0.66 ± 0.07	0.93 ± 0.09	0.69 ± 0.08	0.93 ± 0.07	0.70 ± 0.06	0.95 ± 0.07
Rate body weight/heart (%)	0.26 ± 0.03	0.28 ± 0.03	0.27 ± 0.04	0.27 ± 0.05	0.28 ± 0.05	0.25 ± 0.03	0.28 ± 0.04	0.24 ± 0.03
Right lung weight (g)	0.55 ± 0.04	0.76 ± 0.06	0.58 ± 0.05	0.75 ± 0.07	0.57 ± 0.06	0.84 ± 0.06	0.59 ± 0.04	0.81 ± 0.07
Rate body weight/right lung (%)	0.22 ± 0.03	0.23 ± 0.02	0.24 ± 0.02	0.23 ± 0.03	0.23 ± 0.02	0.22 ± 0.04	0.24 ± 0.01	0.22 ± 0.02
Left lung weight (g)	0.34 ± 0.03	0.45 ± 0.04	0.33 ± 0.03	0.43 ± 0.03	0.34 ± 0.02	0.47 ± 0.04	0.34 ± 0.03	0.48 ± 0.03
Rate body weight/left lung (%)	0.15 ± 0.02	0.14 ± 0.02	0.14 ± 0.01	0.15 ± 0.02	0.14 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	0.13 ± 0.02
Liver weight (g)	8.16 ± 0.40	12.34 ± 0.32	8.21 ± 0.43	12.35 ± 0.35	7.10 ± 0.31	13.58 ± 0.28	6.98 ± 0.29	13.44 ± 0.34
Rate body weight/liver (%)	3.41 ± 0.27	3.77 ± 0.29	3.46 ± 0.22	3.70 ± 0.28	2.92 ± 0.18	3.55 ± 0.22	2.85 ± 0.21	3.55 ± 0.23
Spleen weight (g)	0.52 ± 0.05	0.74 ± 0.04	0.55 ± 0.05	0.75 ± 0.04	0.52 ± 0.04	0.78 ± 0.05	0.50 ± 0.03	0.75 ± 0.05
Rate body weight/spleen (%)	0.21 ± 0.03	0.23 ± 0.03	0.24 ± 0.02	0.23 ± 0.02	0.22 ± 0.03	0.21 ± 0.04	0.21 ± 0.03	0.20 ± 0.02
Pancreas weight (g)	0.26 ± 0.04	0.45 ± 0.05	0.23 ± 0.04	0.47 ± 0.06	0.29 ± 0.03	0.42 ± 0.04	0.26 ± 0.03	0.41 ± 0.04
Rate body weight/pancreas (%)	0.10 ± 0.02	0.14 ± 0.01	0.10 ± 0.02	0.14 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Right kidney weight (g)	0.71 ± 0.07	1.11 ± 0.07	0.69 ± 0.05	1.10 ± 0.08	0.70 ± 0.04	1.09 ± 0.07	0.67 ± 0.03	1.08 ± 0.08
Rate body weight/right kidney (%)	0.29 ± 0.03	0.34 ± 0.02	0.30 ± 0.01	0.33 ± 0.02	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.29 ± 0.01
Left kidney weight (g)	0.73 ± 0.06	1.10 ± 0.08	0.72 ± 0.04	1.08 ± 0.07	0.71 ± 0.03	1.09 ± 0.06	0.70 ± 0.04	1.08 ± 0.07
Rate body weight/left kidney (%)	0.32 ± 0.03	0.34 ± 0.05	0.31 ± 0.02	0.33 ± 0.03	0.29 ± 0.03	0.29 ± 0.02	0.29 ± 0.03	0.29 ± 0.02
Right adrenal gland weight (g)	0.04 ± 0.010	0.04 ± 0.011	0.05 ± 0.011	0.04 ± 0.012	0.04 ± 0.011	0.04 ± 0.013	0.04 ± 0.014	0.05 ± 0.014
Rate body weight/right adrenal gland (%)	0.02 ± 0.012	0.01 ± 0.012	0.02 ± 0.012	0.01 ± 0.012	0.02 ± 0.012	0.01 ± 0.012	0.02 ± 0.013	0.01 ± 0.014
Left adrenal gland weight (g)	0.04 ± 0.012	0.04 ± 0.012	0.05 ± 0.013	0.04 ± 0.013	0.04 ± 0.012	0.04 ± 0.013	0.04 ± 0.012	0.05 ± 0.011
Rate body weight/left adrenal gland (%)	0.02 ± 0.012	0.01 ± 0.014	0.02 ± 0.012	0.01 ± 0.011	0.02 ± 0.013	0.01 ± 0.011	0.02 ± 0.012	0.01 ± 0.011
Right testicle weight (g)		1.72 ± 0.15		1.69 ± 0.13		1.74 ± 0.14		1.74 ± 0.14
Rate body weight/right testicle (%)		0.52 ± 0.03		0.51 ± 0.05		0.45 ± 0.04		0.46 ± 0.03
Left testicle weight (g)		1.74 ± 0.07		1.68 ± 0.08		1.77 ± 0.09		1.73 ± 0.06
Rate body weight/left testicle (%)		0.53 ± 0.04		0.50 ± 0.04		0.46 ± 0.03		0.46 ± 0.03
Bone marrow weight (g)	0.07 ± 0.005	0.08 ± 0.008	0.07 ± 0.007	0.08 ± 0.009	0.08 ± 0.008	0.08 ± 0.011	0.09 ± 0.010	0.09 ± 0.011
Rate body weight/bone marrow (%)	0.03 ± 0.002	0.02 ± 0.002	0.04 ± 0.003	0.02 ± 0.004	0.03 ± 0.003	0.03 ± 0.004	0.04 ± 0.005	0.03 ± 0.003

[a]Data are expressed as mean ± SEM (n = 6 animals/sex group).

V.4.3. Total liver glutathione (GSH) concentration and potential bacteremia

In order to determine changes in the antioxidant defence because of the probiotic treatment, liver GSH concentration was determined. No significant differences in liver GSH concentration were observed between control and treated groups (9.54±1.21 vs 9.37±1.39 mmol/g, P>0.1). This indicates that treatment with *L. salivarius* V4II-90 did not cause oxidative stress to rats and is consistent with the absence of bacteremia since no lactobacilli could be isolated from blood, liver or spleen of the rats. It suggests that the tested strain do not cause either a local or a systemic infection in rats.

V.4.4. Isolation of L. salivarius V4II-90 from feces and vaginal swabs samples

L. salivarius V4II-90 could be isolated from colonic material and vaginal swabs samples of all the treated animals (probiotic groups) at the end of the treatment. The concentration oscillated between 5×10^5 and 7×10^8 cfu/g of colonic material, and between 3×10^3 and 23×10^6 cfu/g in the vaginal swabs. The strain could not be detected in any sample from the placebo group.

V.5. DESIGN OF PCR ASSAY FOR SPECIFIC AND SENSITIVE DETECTION OF *S. agalactiae* IN THE BIOLOGICAL SAMPLES

On the basis of sequence comparison using programs Emma and Showalign, primers *S.agalacFW1* (5´-GTTTGGTGTTTACACTAGA-´3) and *S.agalacRV1* (5´-CAATTGCTCCTTTTAAATAACT-´3) were designed for the specific amplification of a teorethical 148 bp fragment of the 16S rRNA gene of *S. agalactiae* (Figure 11).

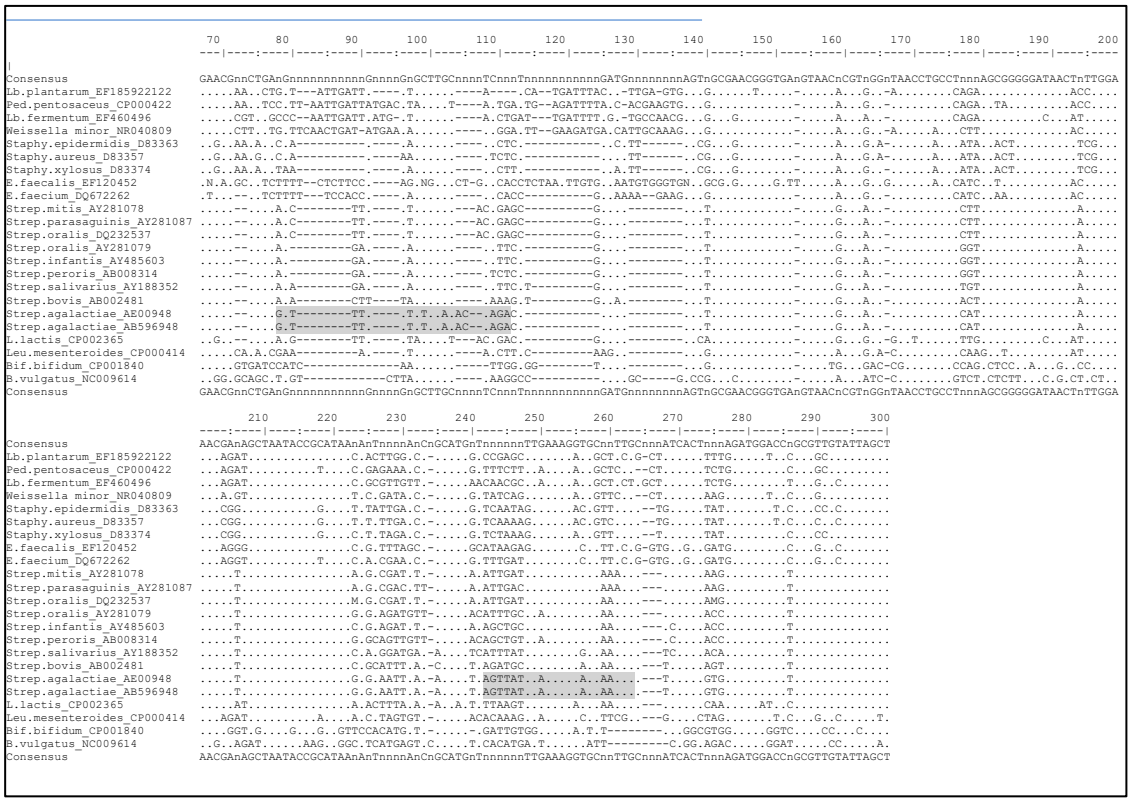


Figure 11. Design of the primers *S.agalacFW1* and *S.agalacRV1*

In conventional PCR, the GBS-specific primer pair (*S.agalacFW1* and *S.agalacRV1*) amplified a single 148 bp amplicon from S. agalactiae DNA. Cross-reactivity was not obtained with DNA from any other species analyzed (Figure 12).

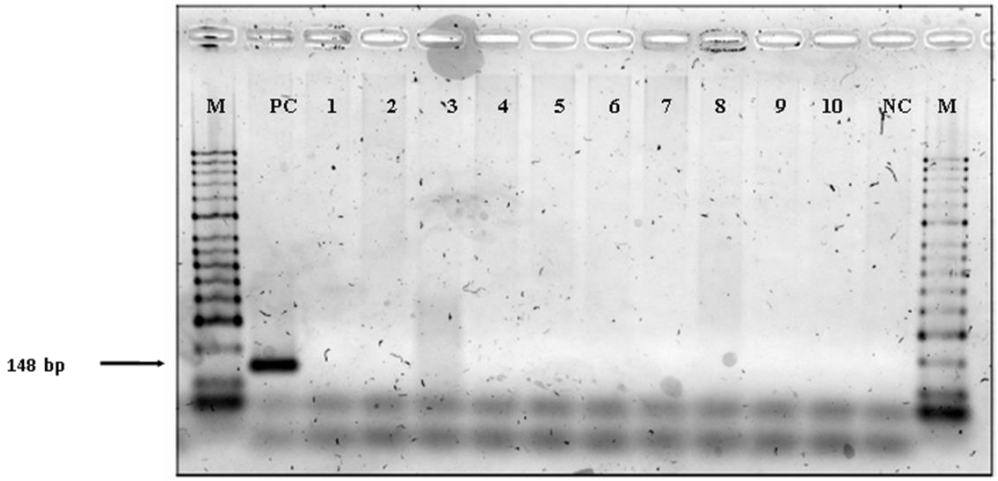


Figure 12. Electroforetic analysis of PCR product of the 148 bp region of *16S rRNA gene* with the use of primers *S.agalacFW1* and *S.agalacRV1* obtained from *Streptococcus peroris* DSM 12493 (lane 1); *Streptococcus mitis* DSM 12643 (lane 2); *Streptococcus oralis* CECT 907 (lane 3); *Streptococcus parasanguinis* DSM 6778 (lane 4); *Streptococcus bovis* DSM 20564 (lane 5); *Streptococcus uberis* DSM 20564 (lane

6); *Streptococcus salivarius* CECT 805 (lane 7); *Streptococcus thermophilus,* ATCC 19987 (lane 8); *Streptococcus pneumoniae* 0566 (lane 9); *Streptococcus dysgalactiae* DSM 20662 (lane 10). M, Molecular weight marker HyperLader™ IV (Bioline); PC, positive control, *S. agalactiae*, DSMZ 2134; NC, negative control. This figure is a reverse image of the agarose gels containing Gel Red™ Nucleic Acid gel Stain 10.000X (Biotium). "bp", base pair.

The real-time specific system amplified a 148 bp fragment from all the *S. agalactiae* samples analyzed. No homologous product was amplified from any other bacterial DNA tested. Threshold cycle (Ct) values between 16.72 and 20.87 were obtained from S. agalactiae DNA. The Ct values measured for DNA extracted from non-target species were 39.82 ± 0.59 (Table 13). Under our experimental conditions, a cut-off value was establish as follows: Ct values above that corresponding to the mean Ct value of all the non-target species and non-template controls minus twice their standard deviation (Ct > 38.64) were considered negative for the presence of target DNA. In relation to the detection limit of this PCR approach, it was observed that the lower percentage of target DNA, the higher Ct values obtained in the real-time PCR with the species-specific primers. The detection limit of this assay was lower than 0.005 ng of target DNA, which corresponds to Ct value of 34.34 (Figure 13).

When real biological samples were tested, there was an excellent agreement between conventional PCR results and those obtained by culture-based methods (Table 14). MALDI-TOF and conventional (qualitative) PCR identification were discordant for only 1 sample. In addition, two samples that were positive by conventional PCR provided a negative result by real-time PCR. Globally, the results obtained indicate that the PCR techniques developed in this study have proven to have a high specificity and sensitivity, and, therefore, they constitute useful GBS screening methods.

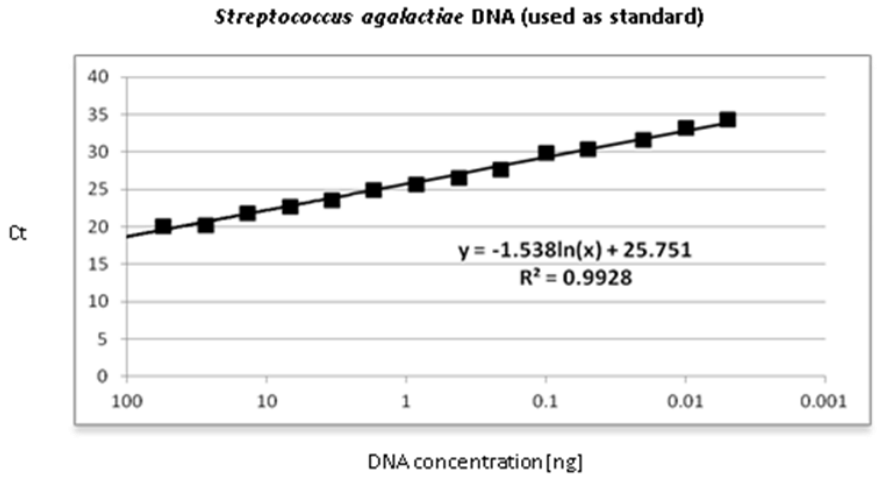
V.6. EFFICACY OF *L. salivarius* V4II-90 TO ERADICATE GBS FROM THE INTESTINAL AND VAGINAL TRACTS OF PREGNANT WOMEN: CLINICAL TRIAL

At the inclusion in the study, GBS was detected in both rectal and vaginal swabs obtained from 39 women, out of a total of 57 participating women, while the rest of women (n = 18) were GBS-negative (Table 15). This last group of GBS-negative women, which did not take a *L. salivarius* strain also had negative GBS cultures from rectal and vaginal swabs taken regularly at 28, 32 and 36-38 weeks (Table 15; Figure 14). A group of GBS-positive women at the start of the study (n = 14) did not receive probiotic and the routine screening results for vaginal and rectal GBS at 28, 32 and 36-38 weeks were found to be all positive (Table 15; Figure 14).

Table 13. Specificity of the real-time PCR system (Ct values obtained from 10 ng DNA).

Scientific name	Crossing point (Ct)	Melting temperature (Tm)
Streptococcus agalactiae DSM 2134	16.77±1.51[a]	80.00
Streptococcus agalactiae M57207	18.08±1.14	80.00
Streptococcus agalactiae M57730	17.97±0.58	80.00
Streptococcus agalactiae M67018	16.72±0.45	80.00
Streptococcus agalactiae M6836	19.07±0.81	80.00
Streptococcus agalactiae M70043	20.87±0.31	80.00
Streptococcus dysgalactiae DSM 20662	40.00±0.00	None
Streptococcus peroris DSM 12493	40.00±0.00	None
Streptococcus mitis DSM 12643	39.01±0.54	None
Streptococcus oralis CECT 907	40.00±0.00	None
Streptococcus parasanguinis DSM 6778	40.00±0.00	None
Streptococcus bovis DSM 20564	38.73±0.51	None
Streptococcus uberis DSM 20564	40.00±0.00	None
Streptococcus salivarius CECT 805	39.83±0.10	None
Streptococcus termophilus ATCC 19987	40.00±0.00	None
Streptococcus pneunoniae 0566	40.00±0.00	None
Bifidobacterium adolescentis HE 005	40.00±0.00	None
Bifidobacterium animalis subsp. *lactis PS17*	40.00±0.00	None
Bifidobacterium breve CECT 4839	40.00±0.00	None
Bifidobacterium infantis CECT 4551	40.00±0.00	None
Bacteroides vulgatus DSM 1447	40.00±0.00	None
Bacteroides fragilis DSM 2151	40.00±0.00	None
Leuconostoc citreum CECT 4025	40.00±0.00	None
Leuconostoc mesenteroides CECT 219	40.00±0.00	None
Leuconostoc fallax LMG 13177	40.00±0.00	None
Weissella cibaria CECT 7032	40.00±0.00	None
Weissella confusa CECT 4707	40.00±0.00	None
Lactobacillus crispatus DSMZ 20584	40.00±0.00	None
Lactobacillus delbrueckii subsp. lactis CECT 282	37.09±1.23	None
Lactobacillus salivarius CECT 4063	40.00±0.00	None
Staphylococcus epidermidis CECT 232	40.00±0.00	None
Staphylococcus aureus CECT 86	40.00±0.00	None
Lactococcus lactis ATCC 10456	40.00±0.00	None
Pediococcus pentosaceus CECT 46595	40.00±0.00	None
Klebsiella pneumoniae subsp. pneumoniae DSM 30104	40.00±0.00	None
Enterococcus faecium CECT 410	40.00±0.00	None
Enterococcus faecalis CECT 481	40.00±0.00	None

[a] Ct values are expressed as mean ± standard deviation



Dilution	*Ct value*	DNA concentration (ng)
10^{-1}	19.24±0.02	110
10^{-2}	20.09±0.15	55
10^{-3}	20.22±0.23	27.5
10^{-4}	21.80±0.07	13.75
10^{-5}	22.64±0.04	6.8
10^{-6}	23.48±0.11	3.43
10^{-7}	24.83±0.07	1.71
10^{-8}	25.66±0.12	0.85
10^{-9}	26.52±0.01	0.42
10^{-10}	27.66±0.04	0.21
10^{-11}	29.82±0.13	0.1
10^{-12}	30.37±0.00	0.05
10^{-13}	31.61±0.10	0.02
10^{-14}	33.18±0.15	0.01
10^{-15}	34.34±0.21	0.005

Figure 13. Detection limit of the real-time PCR system, using ten-fold dilution series of *S. agalactiae* DSMZ 2134 DNA as standard.

Table 14. Results of conventional and real-time PCR assay to the detection of GBS vaginally-colonized women.

Sample	Culture and identification by MALDI-TOF	Conventional PCR amplification results (bp)	Real-time PCR results (Ct)
1	+	148 bp	21.82±0.50 [a]
2	+	148 bp	33.81±0.74
3	-	-	40±0.00
4	+	148 bp	20.85±0.53
5	-	-	40±0.00
6	-	-	40±0.00
7	-	-	40±0.00
8	-	-	40±0.00
9	-	-	40±0.00
10	+	148 bp	22.03±0.53
11	-	-	40±0.00
12	-	-	40±0.00
13	-	-	40±0.00
14	-	-	40±0.00
15	-	-	40±0.00
16	-	-	40±0.00
17	-	-	40±0.00
18	+	148 bp	32.28±0.30
19	-	-	40±0.00
20	-	-	40±0.00
21	-	-	40±0.00
22	-	-	40±0.00
23	-	-	40±0.00
24	-	-	40±0.00
25	+	148 bp	22.01±1.3
26	-	-	40±0.00
27	-	148 bp	35.98±0.97
28	+	148 bp	40±0.00
29	+	148 bp	20.82±1.26
30	-	-	40±0.00
31	-	-	40±0.00
32	-	-	40±0.00
33	-	-	40±0.00
34	+	148 bp	25.30±0.49
35	-	-	40±0.00
36	-	-	40±0.00
37	+	148 bp	18.99±0.31
38	+	148 bp	40±0.00
39	-	-	40±0.00
40	-	-	40±0.00
41	-	-	40±0.00
42	-	-	40±0.00
43	+	148 bp	34.40±1.09
44	+	148 bp	33.01±1.74
45	-	-	40±0.00
46	-	-	40±0.00
47	+	148 bp	30.12±1.66
48	+	148 bp	29.35±0.42
49	-	-	40±0.00
50	-	-	40±0.00
51	+	148 bp	30.16±1.22
52	-	-	40±0.00
53	+	148 bp	24.99±1.69
54	-	-	40±0.00
55	-	-	40±0.00
56	+	148 bp	29.98±1.10
57	+	148 bp	33.70±1.17
58	-	-	40±0.00
59	-	-	40±0.00
60	-	-	40±0.00
61	+	148 bp	27.54±1.00
62	-	-	40±0.00

+ Positive GBS isolation; - Negative GBS isolation/GBS DNA detection.
[a] Ct values are expressed as mean ± standard deviation.

Table 15. Qualitative assessment (GBS positive/GBS negative) of *Streptococcus agalactiae* in rectal and vaginal swabs of participants (N = 57).

Initial GBS status	Probiotic intake		Rectal swabs						Vaginal swabs					
Negative (n = 18)	NO	week	12-26	28*		32**		36-38	12-26	28*		32**		36-38
		GBS positive	0	0		0		0	0	0		0		0
		GBS negative	18	17		16		18	18	17		16		18
		GBS negative (%)	**100**	**100**		**100**		**100**	**100**	**100**		**100**		**100**
Positive (n = 14)	NO	week	14-17	28**		32*		36-38	14-17	28**		32*		36-38
		GBS positive	14	12		13		14	14	12		13		14
		GBS negative	0	0		0		0	0	0		0		0
		GBS negative (%)	**0**	**0**		**0**		**0**	**0**	**0**		**0**		**0**
Positive (n = 25)	YES	week	26	28	30	32	35	38	26	28	30	32	35	38
		GBS positive	25	25	21	12	9	7	25	25	25	15	10	8
		GBS negative	0	0	4	13	16	18	0	0	0	10	15	17
		GBS negative (%)	**0**	**0**	**16**	**52**	**64**	**72**	**0**	**0**	**0**	**40**	**60**	**68**

*: sample from one participant was mising at this sampling time.
**: sample from two participants were mising at this sampling time.

Significantly, the group of GBS-positive women that started using the probiotic (10^9 cfu/daily) since they were enrolled in this study (from 26 weeks) also tested positive for GBS at 28 weeks, but an increasing number of GBS-negative results appeared in the successive swabs collected until delivery (Table 15; Figure 14). At 30 weeks, culture of rectal swabs taken from four women of this group rendered a negative result and the number of these samples increased to 18 (72% of the participants) at 38 weeks. Similar results were obtained culturing vaginal swabs obtained from this group, although the proportion of women testing negative for GBS were always slightly higher when analyzing the rectal swabs than in vaginal swabs (Table 15; Figure 14).

A more detailed analysis revealed that the GBS absence in rectal and vaginal swabs was detected at the same sampling time in 6 participants among women with GBS-negative samples (n = 19) (one woman at 35 weeks and five women at 32 weeks). The detection of GBS absence was most frequently found for the first time in rectal swabs (11 women) than in vaginal swabs (2 women) (Table 16).

The estimation of the concentration of GBS in vaginal swabs taken regularly up to the delivery from all participants is shown in Figure 15. There were no significant changes in both GBS-negative women (n = 18) and GBS-positive women (n = 14) without oral administration of *L. salivarius* V4II-90 regarding the semiquantitative estimation of GBS. However, the number of vaginal swabs where the GBS could not be detected increased in successive sampling times in the group that initially tested positive for GBS taking 10^9 cfu of *L. salivarius* V4II-90 (n = 25). Furthermore, the change in mean bacterial counts for *S. agalactiae* in those women that tested positive and took the *L. salivarius* strain depended both on women and sampling time, but there was no interaction between these two factors (two-way ANOVA; $P<0.001$). The mean value for *S. agalactiae* counts decreased significantly with the administration time *of L. salivarius* V4II-90 (Figures 14 and 15) from a mean value of 5.14 cfu/mL at 26 weeks (n=25) to 3.80 cfu/mL at 38 weeks (n=9) (Figure 15).

Table 16. Comparison of the results obtained for qualitative assessment (GBS positive/GBS negative) of *Streptococcus agalactiae* in rectal and vaginal swabs of participants who tested positive for GBS and received oral administration of *L. salivarius* V4II-90 (10^9 cfu/daily) (n = 25).

	Rectal swab						**Vaginal swab**						**Detection of GBS absence in cultured rectal and vaginal swabs**		
	week						week								
Woman	26	28	30	32	35	38	26	28	30	32	35	38	Simultaneously	For the first time in rectal swab	For the first time in vaginal swabs
3	P	P	P	P	P	P	P	P	P	P	P	P	-		
9	P	P	P	P	P	P	P	P	P	P	P	P	-		
10	P	P	P	P	P	P	P	P	P	P	P	P	-		
17	P	P	P	P	P	P	P	P	P	P	P	P	-		
18	P	P	P	P	P	P	P	P	P	P	P	P	-		
21	P	P	P	P	P	P	P	P	P	P	P	P	-		
15	P	P	P	P	P	P	P	P	P	P	P	N*			week 38
24	P	P	P	P	P	N*	P	P	P	P	P	P		week 38	
6	P	P	P	P	P	N*	P	P	P	P	P	P		week 38	
5	P	P	P	P	N*	N	P	P	P	P	P	N		week 35	
25	P	P	P	P	N	N	P	P	P	P	N	N	week 35		
23	P	P	P	P	N	N	P	P	P	N*	N	N			week 32
7	P	P	P	N*	N	N	P	P	P	P	N	N		week 32	
12	P	P	P	N*	N	N	P	P	P	P	N	N		week 32	
13	P	P	P	N*	N	N	P	P	P	P	N	N		week 32	
22	P	P	P	N*	N	N	P	P	P	P	N	N		week 32	
16	P	P	P	N	N	N	P	P	P	N	N	N	week 32		
19	P	P	P	N	N	N	P	P	P	N	N	N	week 32		
1	P	P	P	N	N	N	P	P	P	N	N	N	week 32		
4	P	P	P	N	N	N	P	P	P	N	N	N	week 32		
8	P	P	P	N	N	N	P	P	P	N	N	N	week 32		
2	P	P	N*	N	N	N	P	P	P	N	N	N		week 30	
11	P	P	N*	N	N	N	P	P	P	N	N	N		week 30	
14	P	P	N*	N	N	N	P	P	P	N	N	N		week 30	
20	P	P	N*	N	N	N	P	P	P	N	N	N		week 30	

Abbreviations: P: positive; N: negative. *: Only one of the swabs (vaginal or rectal) was negative for GBS at that sampling time.

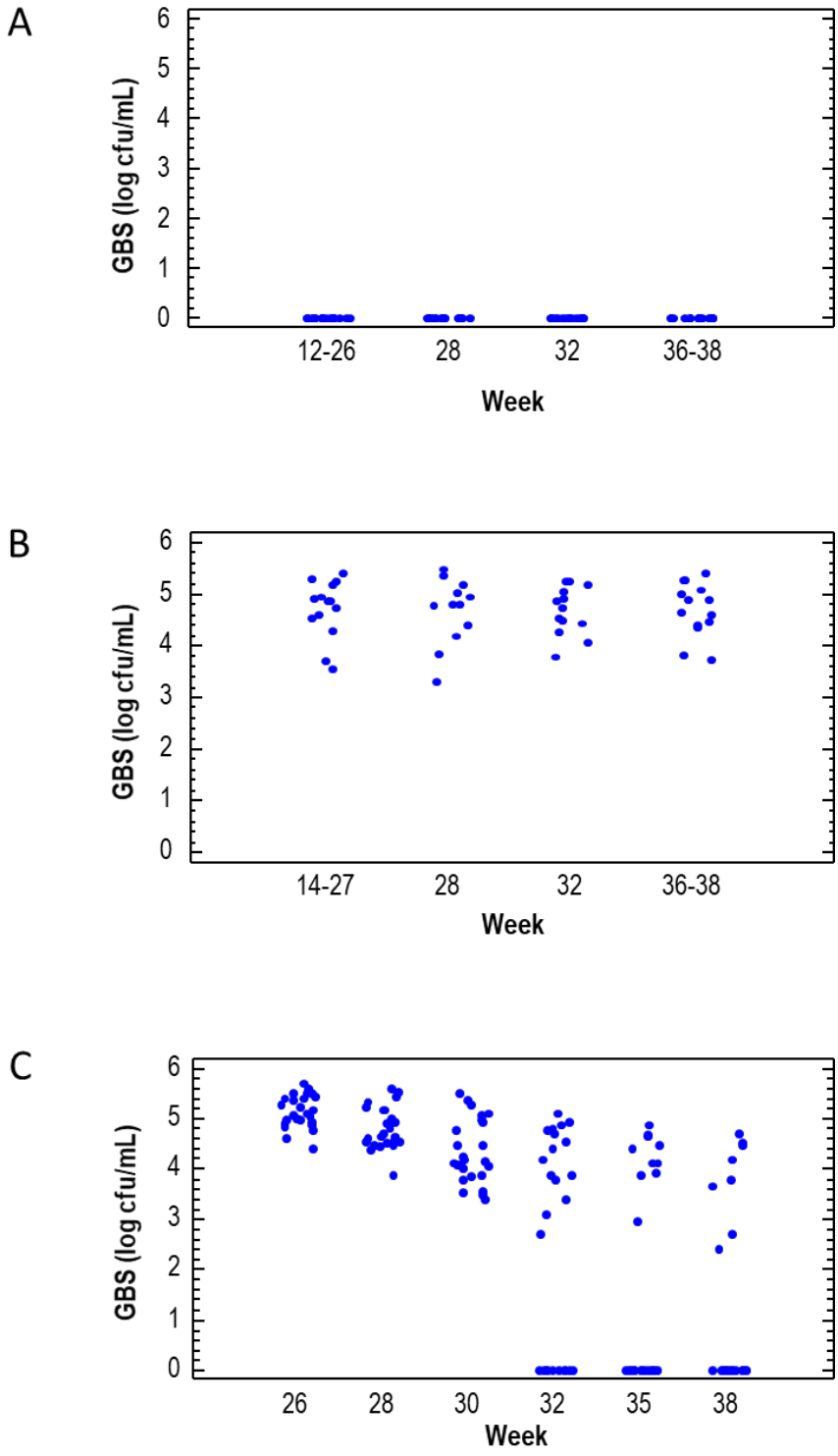


Figure 14. Concentration (CFU/mL) of *S.agalactiae* (GBS) in vaginal swabs taken regularly from A) GBS-negative women (n = 18) without oral administration of *L. salivarius* V4II-90 (10^9 cfu/daily); B) GBS-positive women (n = 14) without oral

administration of *L. salivarius* V4II-90; and C) GBS-positive women (n = 25) in the probiotic group (10^9 cfu of *L. salivarius* V4II-90 daily).

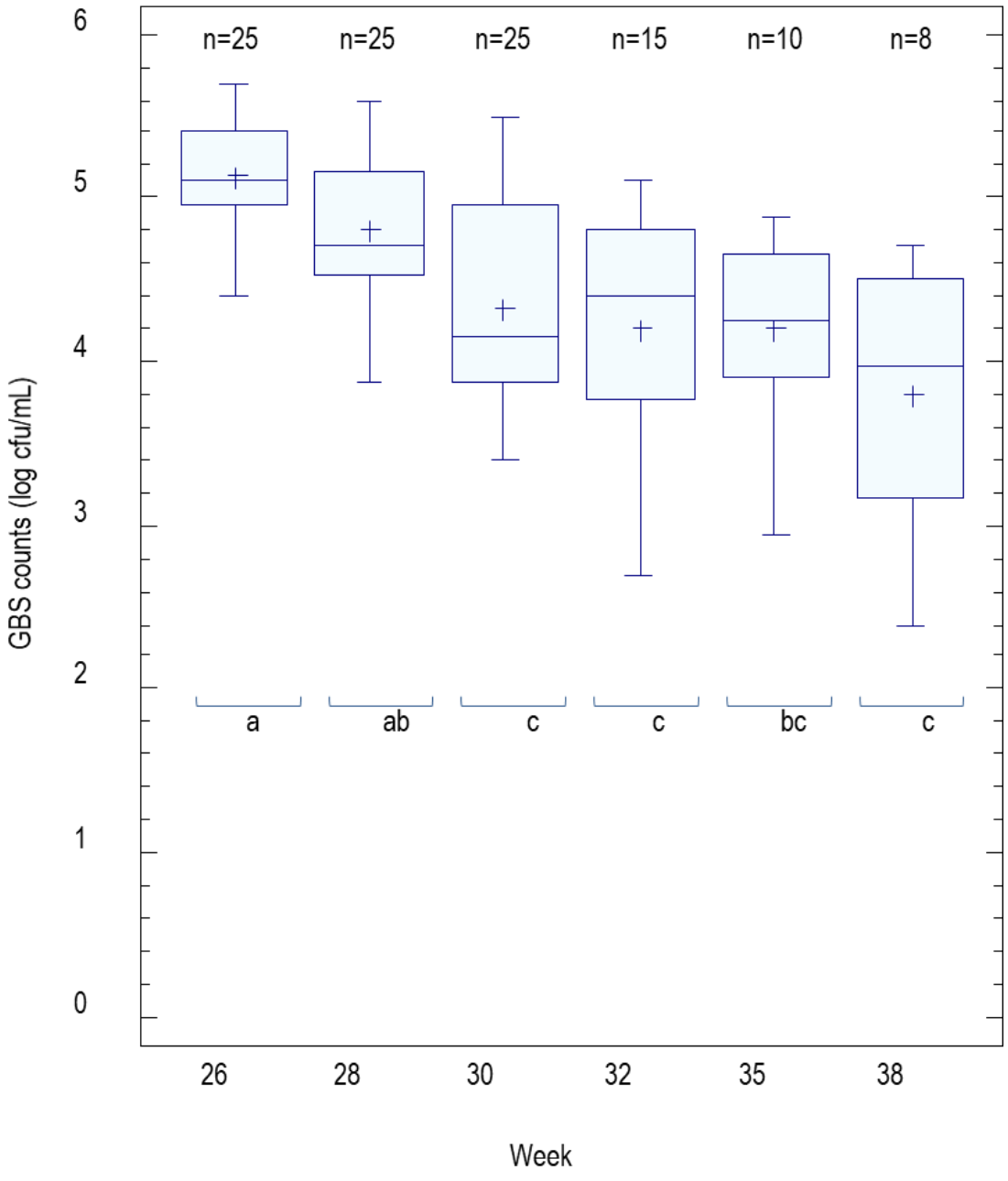


Figure 15. Means concentration (CFU/mL) of *S. agalactiae* (GBS) in vaginal swabs sampled regularly up to the delivery from GBS-positive women taking10^9 cfu of *L. salivarius* V4II-90 daily. Statistically significant differences between samples taking at different sampling times are indicated by letters (Bonferroni post-hoc test).

No adverse effects arising from the intake of *L. salivarius* V4II-90 were reported by any of the women that participated in this study.

VI. DISCUSSION

It is unresolved why GBS establishes as a harmless colonizer in approximately 10% of infants in the first weeks of life, and overcomes epithelial barriers and cellular innate immunity only in less than one in thousand infants to cause LOD. In other words, it remains a puzzle which specific factors at the level of mucosal immunity and the local microbiome allow GBS to leave its colonizing niche, thus facilitating invasion in the individual child.

At the beginning of life, the developmental lines of the microbiota and of the local cellular innate immunity have to run with substantial interdependence. Both areas are subject to factors *in cis* and *in trans*, i.e., specific bacteria are influenced by the microbiota and by host immunity, and host cells are modulated by other host and microbial cells (Thaiss et al., 2016). In order to guarantee long-term ecologic stability, adaptation on either side of the host–microbe interface is required, both at the population level and in the individual cell. The putative contribution of variations in specific innate immune genes to neonatal sepsis has recently been discussed (Borghesi et al., 2017). The authors suggested that affected children may suffer from yet to be identified minor primary immunodeficiency. This is a tempting hypothesis, given the enormous gain in knowledge on single gene alterations leading to susceptibility to a narrow spectrum of microorganisms. On the other hand, there is no indication for inheritance of a specific neonatal sepsis risk. Moreover, LOD typically remains the only "suspicious" episode in the individual infection biography. Finally, preterm birth is a well-recognized risk factor of GBS sepsis. In preterm infants, several factors impact on the individual co-development of microbiota and immunity, in particular cesarean section and formula feeding, which modify the microbiome (Bokulich et al., 2016; Chu et al., 2017), and antibiotic usage, which affects both the microbiome and myeloid cell development (Deshmukh et al., 2014; Josefsdottir et al., 2017)

VI.1. LIMITATIONS OF IAP AS THE STRATEGY TO PREVENT NEONATAL GBS INFECTIONS

The application of universal GBS screening at weeks 35-38 of pregnancy together with IAP during delivery as the general rule for prevention of neonatal GBS infections seems to be an interim strategy due to three main reasons: (l) lack of actual efficacy to reduce the GBS burden, including its inability to prevent mother-to-fetus GBS transmission during pregnancy; (2) contribution to raising antibiotic resistances; and (3) negative impact on the maternal and infant microbiomes. First of all, recent Cochrane reviews have revealed that most of the old randomized controlled trials that supported the adoption of national guidelines for universal GBS recto-vaginal screening in pregnant women (and IAP of those with a positive result) had severe methodological flaws (Ohlsson and Shah, 2009; Ohlsson and Shah, 2013; Ohlsson and Shah, 2014). In fact, the reviews were critical of the overall quality of the studies, particularly the high risk of bias, no a priori sample size calculation, and the failure to use placebo in the trials

against no treatment. The results of these systematic reviews indicated that the use of IAP did not significantly reduce the incidence of all-cause neonatal mortality, neonatal mortality from GBS infection, or from infections caused by bacteria other than GBS. Although the prevalence of GBS infection was reduced with IAP compared to no treatment, the reviews concluded that "*there is lack of evidence from well designed and conducted trials to recommend IAP to reduce EOS by GBS*" (Ohlsson and Shah, 2009). Recent use of antibiotics was associated with a reduced rate of vaginal GBS colonization only when the rectum was not colonized. This observation suggests that either antibiotics were ineffective in eradicating rectal GBS or that they were ineffective at eradicating vaginal GBS among women who were colonized rectally (Meyn et al., 2009). Persistence of GBS colonization with the identical serotype after the use of the recommended or per protocol antibiotics has been reported (Gardner et al., 1979).

VI.2. IAP IN THE CONTEXT OF THE CURRENT ANTIBIOTIC RESISTANCE CRISIS IAP

Secondly, preventive exposure of disproportionally high percentage of mothers and infants to antibiotic may be unacceptable in the future in the frame of the current antibiotic resistance epidemics. This strategy may be more harmful that beneficial in a risk-benefit basis (Michael et al., 2014; Van Boeckel et al., 2014). Antimicrobial resistance (AMR) is an increasingly serious threat to global public health. AMR develops when a microorganism (bacteria, fungus, virus or parasite) no longer responds to a drug to which it was originally sensitive. This means that standard treatments no longer work; infections are harder or impossible to control; the risk of the spread of infection to others is increased; illness and hospital stays are prolonged, with added economic and social costs; and the risk of death is greater—in some cases, twice that of patients who have infections caused by non-resistant bacteria. The problem is so serious that it threatens the achievements of modern medicine. A post-antibiotic era— in which common infections and minor injuries can kill—is a very real possibility for the 21st century (WHO, 2014).

In 1928 a piece of mould fortuitously contaminated a petri dish in Alexander Fleming's Laboratory at St Mary's Hospital London, and he discovered that it produced a substance (penicillin) that killed the bacteria he was examining. Within 12 years Fleming and others had turned this finding into a wonder drug of its time, which could cure patients with bacterial infections. Further antibiotics were discovered and went on to revolutionize healthcare, becoming the bedrock of many of the greatest medical advances of the 20th century. Common yet frequently deadly illnesses such as pneumonia and tuberculosis could be treated effectively. A small cut no longer had the potential to be fatal if it became infected, and the dangers of routine surgery and childbirth were vastly reduced. But bacteria and other pathogens have always evolved so that they can resist the new drugs that medicine has used to combat

them. Resistance has increasingly become a problem in recent years because the pace at which we are discovering novel antibiotics has slowed drastically, while antibiotic use is rising. And it is not just a problem confined to bacteria, but all microbes that have the potential to mutate and render our drugs ineffective (AMR, 2014).

The damaging effects of antimicrobial resistance are already manifesting themselves across the world. Antimicrobial-resistant infections currently claim at least 50,000 lives each year across Europe and the US alone, with many hundreds of thousands more dying in other areas of the world. But reliable estimates of the true burden are scarce. There is considerable variation globally in the patterns of AMR, with different countries often experiencing different major problems. Despite this and in contrast to some health issues, AMR is a problem that should concern every country irrespective of its level of income. For instance, in 15 European countries more than 10% of bloodstream *Staphylococcus aureus* infections are caused by methicillin-resistant strains (MRSA), with several of these countries seeing resistance rates closer to 50%.

Although in modern, well-funded healthcare systems, obtaining access to second and third-line treatments may often not be an issue, mortality rates for patients with infections caused by resistant bacteria are significantly higher, as are their costs of treatment. And we are seeing in parts of Europe an increasing number of patients in intensive care units, hematology units and transplant units who have pan-resistant infections, meaning there is no effective treatment available. The threat of increasingly drug-resistant infections is no less severe in poorer countries. Emerging resistance to treatments for other diseases, such as TB, malaria and HIV, have enormous impacts in lower-income settings. The variation in the AMR problems of individual countries is linked to huge differences in how heavily they use antimicrobial drugs. Global consumption of antibiotics in human medicine rose by nearly 40% between 2000 and 2010, but this figure masks patterns of declining usage in some countries and rapid growth in others (Van Boeckel et al., 2014). Any use of antimicrobials, however appropriate and conservative, contributes to the development of resistance, but widespread unnecessary and excessive use makes it worse. Overuse and misuse of antimicrobials is facilitated in many places by their availability over the counter and without prescription, but even where this is not the case prescribing practices vary hugely between (and often within) countries. Such issues are only made worse by large quantities of counterfeit and sub-standard antimicrobials permeating the pharmaceuticals markets in some regions.

As with all infectious diseases, the speed and volume of intercontinental travel today creates new opportunities for antimicrobial-resistant pathogens to be spread globally. Such mixing of different microbes, particularly bacteria, provides them with opportunities to share their genetic material with each other, creating new resistant

strains at an unprecedented pace. No country can therefore successfully tackle AMR by acting in isolation.

Currently, it is considered that multiresistant bacteria cause more than 700,000 deaths a year in the world, 25,000 of them in Europe (with an additional cost of 1,500 million euros to health systems), according to the data of the Center for Prevention and Control of Diseases of the United States (CDC) and the European Center for Disease Control (ECDC) (Laxminarayan et al., 2013; Laxminarayan et al., 2016). If this trend continues, in 2050 there could be 10 million deaths per year in the world due to this cause and it would constitute the first cause of death worldwide (as a reference it is worth saying that cancer currently causes around 8.2 million deaths per year) (WHO, 2014) (Figure 16). The figures are particularly alarming among the youngest, with an estimate of approximately 214,000 deaths per year due to neonatal sepsis caused by resistant pathogens (Laxminarayan et al., 2016).

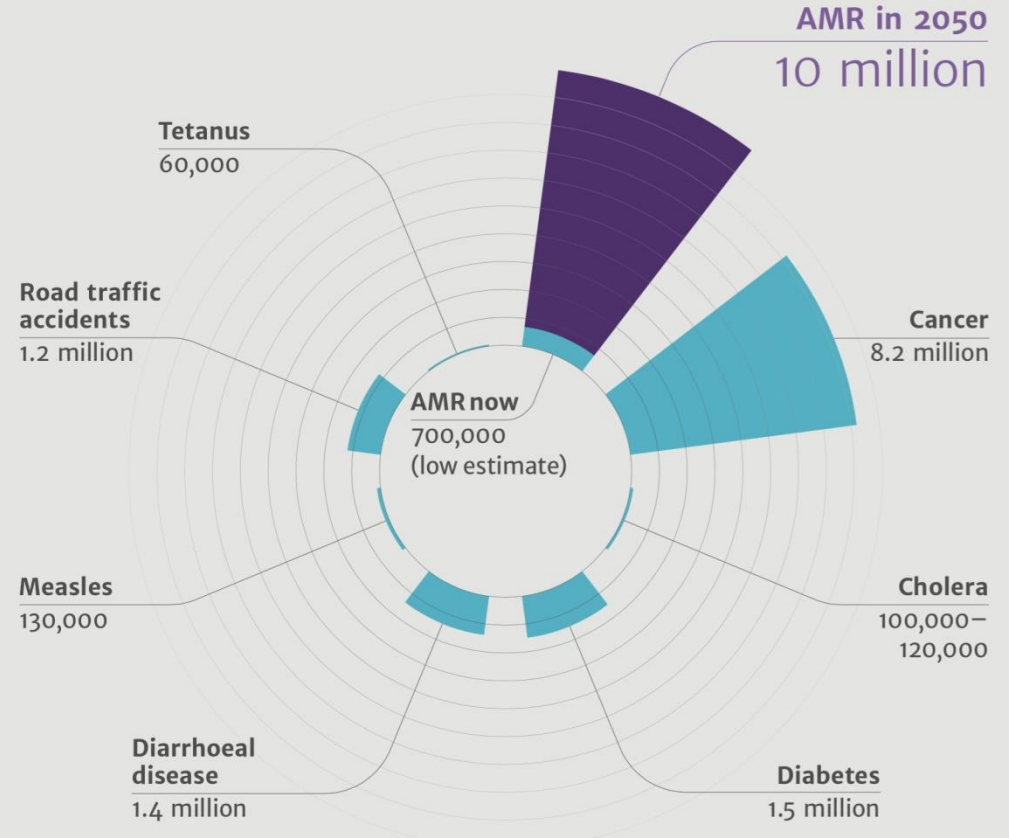


Figure 16. Deaths attributable to antimicrobial resistance every year compared to other major causes of death. Source: AMR (2014).

VI.3. IAP AND THE DEVELOPMENT OF THE HOST MICROBIOME

IAP causes a strong impact on the acquisition and development of the infant microbiota (Tanaka et al., 2009; Aloisio et al., 2016) and it is becoming evident that such an effect has short-, and long term negative consequences for a host health (Blaser, 2016).

After birth, the infant microbiota is characterized by a very low diversity and a high instability in comparison with that of an adult, which makes it especially vulnerable to those factors that, like antibiotics, can modify its normal development (Koenig et al., 2011; Yatsunenko et al., 2012; Goodrich et al., 2014). The vulnerability of the microbiota to antibiotics, and its long-term impact, seems to be greater as the infant age is lower (Schulfer and Blaser, 2015). Paradoxically, the first two years of life is the period in which the use of antibiotics per capita is higher in developed countries (Blaser, 2016); it is estimated that a person receives between 10 and 20 treatments with antibiotics before in his/her first 18 years of life (Sharland, 2007).

Currently, there are two very interesting emerging hypotheses that will have to be carefully evaluated since their implications can be really important in the future. The first, supported by epidemiological and experimental data, is that the effects of antibiotics can be cumulative in an individual (Blaser and Falkow, 2009). Successive antibiotic treatments can lead to the loss of species, especially of taxa that may be in low numbers at that time but may have important metabolic functions in the future. Obviously, the problem would be more relevant in the case of taxa with unique functions. The second, equally relevant, is that the environmental impacts on the microbiome (including exposures to antibiotics, form of birth or diet) are cumulative across generations since, under normal conditions, an individual inherits a large part of the mother's microbiome (Blaser and Falkow, 2009; Sonnenburg et al., 2016) (Figure 17).

The microbiome constitutes an important factor in individual health and development. The composition of the microbiome is complex, distinct between individuals and subject to environmental changes and adaptation to host factors. Each body site contains a unique microbial community. It seems evident that exposure to bacteria in the birth canal impacts on the colonizing microbiota in the infant. However, the fetus may be less sterile than thought, i.e., that the microbiome might develop already *in utero* (Jiménez et al., 2005; Jiménez et al., 2008; Aagaard et al., 2014). Intrauterine colonization data have to be interpreted with some caution, since microbial viability is usually not confirmed and the risk of contamination is high in many of the investigated samples (Lauder et al., 2016; Perez-Muñoz et al., 2017). Accordingly, the contribution of colonization *in utero* to microbiome development is still unclear, whereas that of colonization after rupture of fetal membranes is beyond doubt. As an example, vaginal delivery and cesarean section result in different bacterial communities

on skin, nares, and gingiva (Chu et al., 2017). Yet, the impact of the delivery mode on the expansion and functional diversification after the first 6 weeks of life is surprisingly modest (La Rosa et al., 2014; Chu et al., 2017). Instead, the infant's microbiome follows a rather predictable successive colonization pattern and reaches a stable state resembling the adult microbiome already at 1–3 years of age (Palmer et al., 2007: Koenig et al., 2011; Lozupone et al., 2012). Oxygen abundance in the neonatal gut facilitates the colonization by facultative anaerobes, e.g.,*Lactobacillus* and *Streptococcus*followed by*Enterobacteriaceae*. After oxygen is consumed and anaerobic conditions are established, obligate anaerobic species, e.g.,*Bifidobacterium, Bacteroides*, and *Clostridium*spp. populate the intestine (Adlerberth and Wold, 2009; Tourneur and Chassin, 2013).

Administration of antibiotics, on the other hand, heavily affects the postnatal microbiome (Penders et al., 2006; Yassour et al., 2016; Bokulich et al., 2016). Postnatal exposure to antibiotics alters the gut microbiome in the first 2–3 years of life by delaying microbiome development and altering phylogenetic diversity, e.g., affecting early colonization with *Lactospiraceae* spp. (Bokulich et al., 2016; Yassour et al., 2016). In addition, antibiotics reduce the stability of the microbiota composition as indicated by an increased variation between consecutive samples as compared to controls (Yassour et al., 2016). Notably, very preterm infants with a gestational age of <33 weeks, who in many cases receive antibiotics within 24 h of birth, showed a 10-fold reduced bacterial diversity in comparison to term infants (Gibson et al., 2016).

VI.4. GBS AS PART OF THE HUMAN MICROBIOME

Streptococcus is, together with *Lactobacillus, Staphylococcus*, and *Propionibacterium*, one of the most commonly found bacterial genera in the neonatal intestine and oral cavity (Jiménez et al., 2008a; Chu et al., 2017). Streptococcal species account for up to 10% of total bacteria in fecal samples during the first months of life (Arboleya et al., 2015; Heida et al., 2016; Mazzola et al., 2016). In pregnant women, GBS colonization is found in up to 30% of rectovaginal samples (Hansen et al., 2004; Stoll et al., 2011) and stable colonization with the same clone for several years has been demonstrated (Hansen et al., 2004). Spread from the gastrointestinal tract to the genital tract is considered to be a probable colonization sequence for GBS (Dillon et al., 1982). Since strains might be lost or reacquired in relatively short time periods (Manning et al., 2008; Kwatra et al., 2014), GBS screening is recommended relatively late in pregnancy, i.e., between gestational weeks 35 and 37 (Verani et al., 2010).

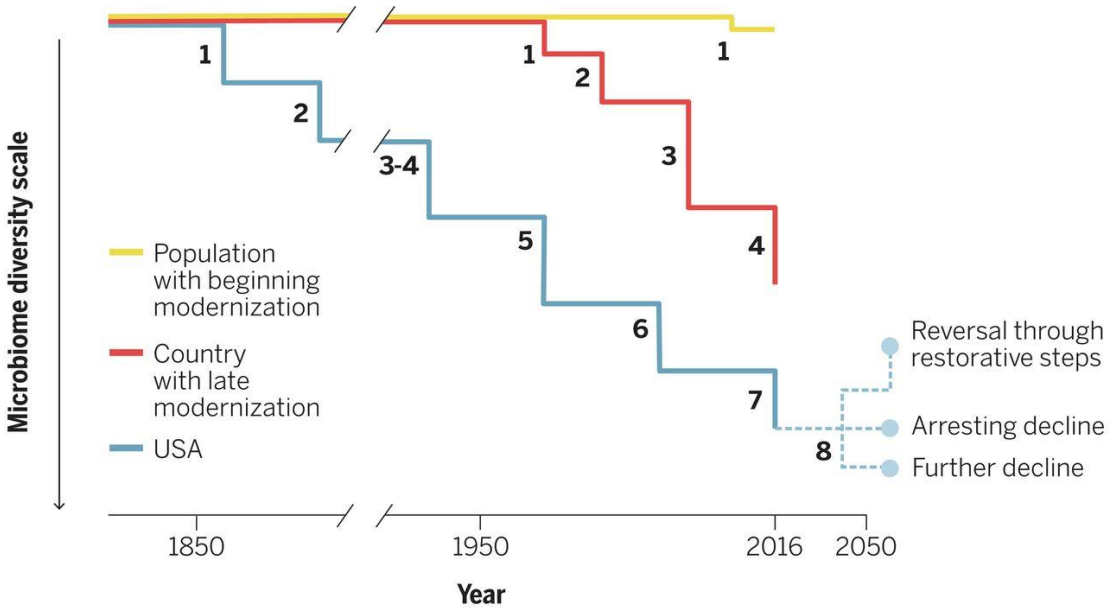


Figure 17. Models of microbiota change in different societies. The decline in microbiota diversity in the United States happened simultaneously with the early introduction of sanitation, including filtered and chlorinated drinking water, and early antibiotic use. The scale is arbitrary and reflects the aggregate of species and strain losses. The numbers shown represent the generations since the earliest population-wide microbiota species and strain losses and show progressive and cumulative loss of diversity. Each line represents an average; within every generation, there is variation in individual positions, based on their founding populations, exposures, and timing. In a country with late modernization, the diversity loss occurred later, but generation times are shorter, and the steps more irregular and increasing, which reflects the effects of the accelerated pace of modernization in recent years on human microbiota biodiversity loss in developing countries. For the future, three trajectories are shown for the developed country. Source: Blaser (2016).

Colonization by GBS is not exclusively confined to humans. Instead, GBS was first described in the 1880s as a cause of mastitis in goats and cows and it is a frequent commensal in seals and fish (Manning et al., 2008; Delannoy et al., 2013). Although rare, invasive GBS disease can be a zoonotic disease as outbreaks in adults have been linked to raw fish consumption (Rajendram et al., 2016). Moreover, the hypervirulent ST-17 strain, which emerged 40 years ago, shares greater genetic similarity with bovine than with many human strains, indicating that it originated from a bovine lineage. Therefore, GBS may—under very specific conditions—cross species barriers (Bisharat et al., 2004). However, since virulent strains in humans are distinct from those causing disease in animals (Manning et al., 2008; Bohnsack et al., 2008), person-to-person transmission plays the primary role in human GBS dissemination. Data on GBS spread are largely confined to mother-infant pairs. In contrast, the contribution of fecal-oral transmission by other family members than the mother to GBS colonization of the infant

remains unclear. While strains are largely shared between sexual partners (Manning et al., 2002; Meyn et al., 2002), cohabitation appears to play a minor role in transmission (Manning et al., 2004).

Intrapartum antibiotic prophylaxis during delivery may transiently increase the GBS colonization risk of the infant yet probably does not affect the relative abundance of *Streptococcus* spp. in the stool beyond the first few weeks of life (Manning et al., 2008). While a number of studies longitudinally analyzed the development of the microbiome after birth on the level of phylum, class or order, studies on species or even genus level, e.g., with a specific focus on Group A *Streptococcus* (GAS) or GBS are rare and do not allow for robust statements on this level of resolution. Infants which were tested negative for GBS after IAP administration frequently acquire maternal GBS strains at later time points (Berardi et al., 2013). Breast milk is hence a probable source of GBS in LOD. Some LOD case studies detected GBS in breast milk (Filleron et al., 2014). However, it is often unclear whether GBS in breast milk results from maternal colonization or infant oropharyngeal contamination. Mutated strains from infants which have been detected in the maternal breast milk (Almeida et al., 2015) support the latter hypothesis.

In addition, most breastfed infants remain unaffected by GBS in breast milk and, in fact, some researchers consider that human milk may protect neonates from GBS infections (Le Doare and Kampmann, 2014). Mechanisms associated with transmission of GBS in breast milk and potential factors that may protect the infant from transmission remain poorly understood. Understanding factors involved in protection or transmission of GBS infection via breast milk is important both for premature infants who are a high-risk group and for infants in the developing world where breastfeeding is the only sustainable infant feeding option. There is increasing data from recent publications that enhanced protection against diarrhea, respiratory tract infections, otitis media and *H. influenzae* infections, as well as wheezing illness may persist for years after breastfeeding. However, the role of breast milk antibody in protection from neonatal GBS disease remains poorly understood. Breast milk provides considerable amounts of specific SIgA antibodies that are produced as a result of microbial and food antigens the mother has previously encountered. Such SIgA antibodies from breast milk provide protection to the neonate at the mucosal surface. Breast milk additionally contains high concentrations of non-specific protective molecules, such as lactoferrin that has bactericidal, viricidal, and fungicidal properties. Milk oligosaccharides might block adherence of microorganism at the mucosal surface by functioning as receptor analogues.

Although GBS is the most prevalent streptococcal strain in neonatal sepsis, other streptococci, notably Groups A, D, and G, are isolated from blood cultures of newborns as well (Berkley et al., 2005; Talbert et al., 2010). Indeed, the connection of GBS and

neonatal sepsis was only found in the 1960s and its predominance was established in the 1970s (Bisharat et al., 2004; Da Cunha et al., 2014). Prior to that, GAS and *Streptococcus pneumoniae* accounted for most neonatal sepsis cases (Bizzarro et al., 2005; Shah and Padbury, 2014). As in other ecological niches, competition for nutrition and space occurs between bacterial species on colonized human body sites (Hibbing et al., 2010). Indeed, examples of mutual exclusion are found in the genus *Streptococcus*, e.g., in the case of *Streptococcus mutans*, the predominating cause of caries. The presence of other streptococcal species in the oral cavity, namely *Streptococcus sanguinis* and *Streptococcus oligofermentans*, is inversely correlated with the abundance of *S. mutans* which has been linked to the production of hydrogen peroxide *in vitro* (Kreth et al., 2005; Tong et al., 2007). Another example is the observation that *Corynebacterium* and *Dolosigranulum* in the upper respiratory tract are protective against colonization with *Streptococcus pneumonia*, which causes otitis media in infants after colonization of the airways (Laufer et al., 2011). More importantly in the context of this review, growth of GBS is inhibited by *Streptococcus salivarius* both *in vitro* and in a vaginal colonization mouse model (Patras et al., 2015). Competitive growth was also shown for *Bifidobacterium* and GBS *in vitro* (Aloisio et al., 2014) and lactobacilli inhibited growth (Ruiz et al., 2012) and attachment of GBS to vaginal epithelial cells (Zarate et al., 2016). In addition, *Lactobacillus reuteri* reduced vaginal colonization in a mouse model (De Gregorio et al., 2015) and—importantly—as a probiotic in a placebo-controlled trial in pregnant women (Ho et al., 2016). These findings are in line with a very recent randomized, double-blind, placebo-controlled trial from Indian, where *Lactobacillus plantarum* plus fructooligosaccharides protected newborns from sepsis (Panigrahi et al., 2017). In general, however, the presence of GBS appears not to be linked to an abnormal microbiome or a reduction of the predominant *Lactobacillus* genus in the vaginal tract of the mother (Brzychczy-Wloch et al., 2014; Rick et al., 2017; Rosen et al., 2017). Interestingly, a small study found significant taxonomic differences in stools of 6-month infants, when mothers were GBS carriers, as compared to non-carriers (Cassidy-Bushrow et al., 2016). Yet, robust epidemiological evidence for a correlation of neonatal colonization with GBS and that of other specific intestinal commensals such as other streptococcal species is not existent.

Next to streptococci, staphylococci cause bacteremia and sepsis in newborns. Indeed, coagulase-negative staphylococci are the most common cause of nosocomial sepsis in newborns, yet do not play a role in healthy term infants. The generally more virulent *S. aureus* is isolated in variable frequency from neonatal blood cultures, but it is rarely found in cerebrospinal fluid (Talbert et al., 2010). Furthermore, in view of the omnipresence of *S. aureus* as a colonizer in up to 50% of neonates, infants of this age group are not specifically susceptible to staphylococcal infections, unless they are subject to medical interventions such as indwelling catheters or surgery (Lowy, 1998; Berkley et al., 2005). Hence, the contact with GBS and potentially other (beta-

hemolytic) streptococci and the establishment of coexistence with these bacteria appears to impose a greater risk to the infant compared to other genera.

VI.5. THE IMPACT OF ANTIBIOTIC PRESSURE AND RESISTANCE ON GBS-RELATED LOD

The majority of GBS strains isolated from humans are resistant to the antibiotic tetracycline. Indeed, the insertion of tetracycline resistance (TcR) elements, i.e., the ribosomal protection proteins Tet(M) and Tet(O), in few GBS clones led to their selection and expansion after the onset of extensive tetracycline usage since 1948 (Da Cunha et al., 2014). These clones have since replaced a prior diverse GBS population, concurrent with the rise of GBS as major cause of neonatal sepsis. Notably, TcR elements are the most widely spread resistance genes in the human gut microbiota (Hu et al., 2013). Moreover, a subset of GBS strains, especially ST-1, carry genes which confer general resistance to macrolids and lincosamides, i.e., the methylases erm(B) and erm(TR) (Da Cunha et al., 2014). Resistance rates to clindamycin (lincosamid) and erythromycin (macrolide) range up to 30 and 50%, respectively (Hays et al., 2016; Teatero et al., 2016). A rise of resistance to fluoroquinolones has been described in serotype V strains (Kimura et al., 2013; Hays et al., 2016). In addition, GBS with reduced penicillin susceptibility due to mutations in the penicillin-binding proteins are isolated with increasing frequencies in Japan (Kimura et al., 2008; Seki et al., 2015) and were also reported to occur spontaneously in an American patient after prolonged penicillin treatment (Longtin et al., 2011). In this context, it seems likely that the frequent use of antibiotics other than tetracyclines may also lead to selection of hypervirulent strains. In the Netherlands, the incidence of EOD caused by ST-17 has significantly increased after implementation of a risk-based approach of antibiotic prophylaxis (Bekker et al., 2014). ST-17 strains are also significantly more prevalent in women with IAP as compared to other strains (Manning et al., 2008). Thus, a relatively short course of intrapartum antibiotics, usually penicillin and ampicillin, may allow for seeding and expansion of hypervirulent GBS strains, which may not affect the majority of infants but propagate LOD development in few colonized individuals.

In addition, the capsular serotypes of GBS are not fixed but subject to frequent exchange by conjugative transfer between strains, explaining for the diversity of serotypes within clonal complexes. Lately, serotype IV has emerged as a causative agent of adult GBS disease in the USA (Teatero et al., 2015; Teatero et al., 2016). This seems important, as serotype IV is not included in the latest efforts in vaccine development to capsular antigens of GBS. Sequencing has revealed that a predominating serotype IV strain acquired large genomic fragments by horizontal gene transfer from the hypervirulent ST-17 and ST-23 strains (Campisi et al., 2016). Additionally, ST-17 strains with capsular switching to serotype IV have been identified in several countries (Bellais et al., 2012; Meehan et al., 2014). Since maternal antibodies can impact on

colonization with the antibody-specific GBS strains in mothers and early infants (Le Doare and Kampmann, 2014; Kwatra et al., 2015; Baker et al., 2017), it remains an open question whether targeting certain serotypes may eventually select for strains which have acquired novel capsule genes and allow for their expansion.

Interestingly, single-nucleotide polymorphisms (SNPs) in virulence-associated genes were detected in neonatal invasive GBS strains in comparison to the respective colonizing strains from the mothers, possibly contributing to the transition from a maternal commensal to a neonatal pathogen (Almeida et al., 2015). This suggests that mutations are positively selected for in the neonatal environment. Moreover, mutations in the virulence regulator CovR/S leading to hyperhemolytic activity were found in invasive isolates of women in preterm labor (Whidbey et al., 2013). The acquisition of antibiotic resistance, serotype switching and SNPs can therefore lead to microevolution in the individual newborn, which may explain the pathogenicity of GBS in only a very small number of infants.

VI.6. THE ROLE OF ANTIBIOTICS AND DYSBIOSIS IN THE DEVELOPMENT OF GBS SEPSIS

The microbiota may have beneficial but also detrimental, acute, and chronic effects on infant health. Dysbiosis may predispose the neonatal intestine to inflammation (Tourneur and Chassin, 2013) and facilitate the expansion of otherwise infrequent pathobionts (Ayres et al., 2012; Stecher et al., 2013). Dysbiosis with lower bacterial diversity and decreased density of *Propionibacterium* spp. was found to precede the onset of necrotizing enterocolitis (NEC) (Morrow et al., 2013; Stewart et al., 2016). Moreover, lactate-producing bacilli such as staphylococci and streptococci were reduced after birth in infants with NEC (Mazzola et al., 2016). Even though the increased prevalence of opportunistic pathogens such as uropathogenic *E. coli* (Ward et al., 2016) and *Clostridium perfringens* (Mazzola et al., 2016) has been linked to NEC, a common bacterial signature has not been found (Stewart et al., 2016; Pammi et al., 2017). In addition, it is often unclear whether dysbiosis and the development of organ pathology are causally linked or whether they both depend on upstream disturbances, which may be diverse. Mai et al. found signs of dysbiosis in preterm infants already 2 weeks before onset of sepsis (Mai et al., 2013). Dysbiosis meant a delayed colonization with *Proteobacteria* and decreased density of *Bifidobacteria* spp. This observation receives support by the finding that *Bifidobacterium* spp. in the gut are protective for LOD (Stewart et al., 2017), although the data on this issue are not fully consistent between studies (Taft et al., 2015). In view of these observations, a reduced intestinal *Bifidobacterium* density in infants whose mothers received IAP constitutes an important warning sign for the most careful usage of antibiotics in this sensitive period (Aloisio et al., 2014). In support of this notion, the risk for LOD caused by various pathogens including GBS in preterm infants is threefold higher after prolonged empirical antibiotic treatment (Kuppala et al., 2011). Antibiotics can affect the composition of the

microbiome in many ways, including the depletion of competitive microbes, a delay in immune cell maturation (see below) and dysbiosis, all of which widen the niche for pathogenic bacteria.

VI.7. GBS COLONIZATION RATES

In this PhD Thesis, the GBS colonization rates were 25% and 20% among non-pregnant and pregnant women, respectively. Globally, there is a limited knowledge of GBS colonization prevalence and risk. While regional GBS vaginal colonization rates have been estimated, colonization rates in many individual countries, including Spain, are unknown. Estimates of GBS vaginal colonization in different countries can be highly variable, with interstudy rates differing by as much as 20% (Hillier et al., 1991). This variability may be due to differences between subregions of large countries (e.g., India) or the means of diagnosing colonization (culture-based methods vs. PCR-based methods vs. serology-based methods). More comprehensive country-based and subregional-based studies are necessary to fully understand the burden of GBS colonization. New studies should focus on areas where preterm birth rates and neonatal mortality rates are especially high, such as sub-Saharan Africa or south Asia. Additionally, few studies provide information about GBS serotype prevalence, colonization load, antibiotic-resistance profiles, or valuable genetic information, such as virulence gene prevalence.

While diagnostic technologies exist to evaluate these indicators, they can be time-consuming, expensive, technically challenging, and overall impractical. Diagnostic methodology for GBS colonization has not advanced as rapidly as our understanding of the disease itself, and new technologies need to be developed to garner more information from future studies to further refine our knowledge of GBS colonization. Finally, more studies need to be designed to conclusively identify risk factors for GBS colonization, ascending infection, and GBS-associated preterm birth. It is clear that previous GBS colonization is a risk factor for colonization during a subsequent pregnancy (Allen et al., 1999), but few risk factors for initial GBS colonization have been identified. Recent studies have identified obesity (Lawn et al., 2010) and black ethnicity as possible risk factors for colonization (Romero et al., 2014). Future studies should be designed to identify population characteristics beyond ethnic demography and age of GBS-colonized women in an effort to improve our ability to identify at-risk individuals. Ultimately, universal screening programs are needed in more countries to measure the burden of GBS colonization and successfully prevent disease.

Recently, significant effort has been dedicated to measuring the global rates of GBS colonization, invasive disease, and related risk factors. In the USA and many other countries (including Spain), women are routinely screened in the late third trimester (between 35 and 37 weeks' gestation) for GBS colonization by rectovaginal swab and subsequent culture. If the rectovaginal swab is culture-positive, or if the patient has GBS

in the urine, or has a prior history of GBS perinatal infection, intrapartum prophylactic antibiotics are administered to prevent vertical transmission of GBS to the neonate during labor and delivery. Unlike the USA, some European countries (e.g., UK) have not adopted the GBS screening program but instead administer antibiotics upon the development of a risk factor for GBS neonatal disease (e.g., prolonged rupture of membranes). However, none of these approaches have eliminated neonatal GBS infections. This is because these prevention strategies do not address the risk of ascending infection, which can potentially occur anytime during pregnancy, leading to preterm birth or stillbirth. Also, these approaches do not prevent late-onset GBS infections (observed in neonates who are older than 1 week of age) where vertical transmission is not the only mode of acquisition.

Overall, prevention of GBS infection in pregnancy is still a complex question, with risk likely imparted by several factors, including: pathogenicity of the GBS strain, host factors, influence of the vaginal/rectal microbiome, false-negative screening results, and/or changes in GBS antibiotic resistance. As current interventions targeting GBS infections are limited to antibiotic therapy, and given that antibiotic resistance is on the rise, a deeper understanding of how GBS is able to colonize the vagina and cause neonatal disease is critical for the development of new therapeutics. Recently, a number of studies have described host and bacterial factors important for GBS infections during pregnancy.

Currently, strategies are mainly focus on the prevention of GBS transmission during labor and delivery through the use of antibiotics. This strategy does not fully capture the biology of GBS infection, nor does it completely address the full burden of GBS disease. Moreover, antibiotic resistance is increasing, and the use of antibiotics during pregnancy has consequential effects for neonatal health that are only now being appreciated (Bokulich et al., 2016). To successfully eradicate the burden of disease, interventions need to be specifically targeted, have minimal detrimental effects on the microbiome, and target processes upstream of vertical transmission, such as colonization and ascending infection. Multiple studies have focused on a probiotic approach to reducing vaginal GBS colonization. This issue will be discussed in the next section.

VI.8. SEARCHING FOR ALTERANTIVES TO IAP: GBS-TARGETING PROBIOTIC

As stated above, there is a need for alternatives that are respectful with the neonatal and infant microbiota, and that do not compromise the health of future generations. In this context, the final objective of this work was the selection of safe probiotic strains with the in vitro and in vivo ability to eradicate GBS from the intestinal and genitourinary tracts of pregnant women and/or their infants.

The genus *Lactobacillus* constitutes the dominant bacterial group of the vaginal tract in most healthy women, playing a key role in the genitourinary homeostasis (Boris and Barbés, 2000; Reid and Burton, 2002; Ravel et al., 2011; Ma et al., 2012). In addition, this genus is also commonly found in the gastrointestinal tract. As a result, the selection and characterization of lactobacilli strains to be used as probiotics in a very wide array of gastrointestinal and genitourinary conditions has been a hot topic in Biomedicine for the last years, with hundreds of reviews and *in vitro* and *in vivo* original work articles being published each year. *Lactobacillus* strains have been proposed for use in women before, during and after pregnancy (Fernández et al., 2014; Reid et al., 2016).

In this study, all the vaginal isolates (from either pregnant or non-pregnant healthy women) that fulfilled the initial selection criteria belonged to the same species: *L. salivarius*. This species is part of the indigenous microbiota of the human gastrointestinal tract, oral cavity, genitourinary tract and milk (Rogosa et al. 1953; Casey et al. 2004; Martín et al., 2006; Al Kassaa et al., 2014; De Gregorio et al., 2014), and some strains have been studied as probiotics because of their *in vitro* and *in vivo* antimicrobial, antiinflammatory and immunomodulatory properties (Dunne et al., 1999; Mattila-Sandholm et al., 1999; Dunne et al., 2001; McCarthy et al., 2003; Dunne et al., 2004; Sheil et al., 2004; Martín et al., 2006; Olivares et al. 2006; Pérez-Cano et al., 2010; Neville and O'Toole, 2010; Langa et al., 2012; Messaoudi et al., 2013; Sun et al., 2015).

Administration of probiotic bacteria benefits the host through a wide array of mechanisms that are increasingly recognized as being either species- and/or strain-specific (Hill et al., 2014). A comparative genomics study that included 33 *L. salivarius* strains isolated from humans, animals or food revealed that this species displays a high level of genomic diversity (Raftis et al. 2011). Therefore, selection of *L. salivarius* strains for probiotic use requires pragmatic experimental validation of target-tailored phenotypic traits. Some *L. salivarius* strains have shown to be efficient to prevent infectious diseases, such mastitis caused by staphylococci and streptococci, when administered during late pregnancy (Fernández et al., 2016). Moreover, oral administration of *L. salivarius* strains is also a valid strategy for the treatment of such condition during lactation and, in fact, one of the strains was more efficient that antibiotics for this target (Arroyo et al., 2010; Vázquez-Fresno et al., 2014; Espinosa-Martos et al., 2016). In this PhD Thesis, the target was antagonism towards GBS and, as a consequence, properties such as antimicrobial activity against *S. agalactiae* strains or coaggregation with this species were considered particularly relevant.

The production of antagonistic substances such as bacteriocins, hydrogen peroxide or organic acids represents an important contribution to the defense mechanisms exerted by intestinal and vaginal lactobacilli (Martín et al., 2006; Martin et al., 2008). Some *L. salivarius* strains produce bacteriocins or display bacteriocin-like activity against a

variable spectrum of Gram-positive bacteria (Ocaña et al., 1999; Flynn et al., 2002; O'Shea et al., 2012), including *S. agalactiae* strains (Ruiz et al., 2012). In our study, none of the selected *L. salivarius* strains displayed bacteriocin-like activity against *S. agalactiae* strains. Future antimicrobial assays and/or analysis of the *L. salivarius* V4II-90 will determine if this strain produces bacteriocin/s with activity against other bacterial species. Therefore, the antimicrobial activity that the selected *L. salivarius* strains exhibited against *S. agalactiae* must be related with the production of other antimicrobial compounds, such as organic acids. The ability of lactobacilli to acidify the vaginal milieu contributes to the displacement and inhibition of pathogens proliferation (Charlier et al., 2009) and, more specifically, acid production by lactobacilli has been directly correlated with the inhibition of GBS growth (Açikgöz et al., 2005). Another antimicrobial defense mechanism attributed to some intestinal or vaginal lactobacilli is the production of peroxide hydrogen, a compound that is toxic for catalase-negative bacteria, such as streptococci (Borges et al., 2014). Production of this compound by *L. salivarius* has already been reported (Ocaña et al., 1999; Martín et al., 2006). In our study, *L. salivarius* V4II-90 (the strain that showed the highest anti-GBS activity) produced high amounts of lactic acid and, in addition, was able to produce peroxide hydrogen.

The ability to adhere to intestinal or vaginal epithelial cells or to mucin, and to co-aggregate with potential pathogens constitutes one of the main mechanisms for preventing their adhesion and colonization of mucosal surfaces. Therefore, it is not strange that such properties are considered relevant for the selection of probiotic strains (Reid et al., 1988; Younes et al., 2012). High adherence of *L. salivarius* strains to Caco-2 and HT-29 cells or to mucin has been previously observed (Dunne et al., 1999; Martín et al., 2006; Martín et al., 2009). Globally, *L. salivarius* V4II-90 showed the best combination of adherence to epithelial cells, co-aggregation with *S. agalactiae* and inhibition of *S. agalactiae* strains in broth co-cultures. This strain showed a high survival rate during transit through an in vitro gastrointestinal model, similar to those obtained with other *L. salivarius* strains using the same model (Martín et al., 2006; Martín et al., 2009). Survival of lactobacilli when exposed to conditions found in the gastrointestinal tract seems to be a critical pre-requisite for a probiotic strain when use as a food supplement is pursued, as it was the case.

Some vaginal strains of *L. gasseri* and *L reuteri* have also been reported to co-aggregate with GBS (De Gregorio et al., 2014). In contrast, no co-aggregation activity between *S. agalactiae* and other vaginal lactobacilli belonging to the species *L. acidophilus, L. gasseri* and *L. jensenii* was observed in other study (Boris et al., 1998), a fact suggesting that such property is a highly strain-specific trait (Collado et al., 2007; Ekmekci et al., 2009) related with cell surface components and influenced by different factors (Boris et al., 1997; Boris et al., 1998; Ocaña and Macías, 2002). In relation to broth co-cultures, the capacity to antagonize the growth of *S. agalactiae* by lactobacilli strains belonging to different species, including *L. salivarius*, has been previously

reported (Bodaszewska et al. 2010; De Gregorio et al., 2014). Similarly to our results, this activity was strain-dependent (De Gregorio et al., 2014).

One of the most important criteria for the selection of probiotic strains is the assessment of their safety, particularly to the target population. In this work, no adverse effect was reported by any of the women participated in the clinical trial and ascribed to the probiotic group (thus, receiving *L. salivarius* V4II-90 at 9 $\log_{10}$ cfu daily for several weeks). Previously, other *L. salivarius* strains have been shown to be well tolerated and safe in animal models (Lara-Villoslada et al. 2007) and in human clinical assays (Arroyo et al. 2010; Maldonado et al. 2010; Vázquez-Fresno et al., 2014; Espinosa-Martos et al., 2016), including pregnant women (Fernández et al., 2016). However, safety must be assessed in a strain by strain manner since, although rare, adverse effects due to the consumption of lactobacilli strains have been reported (Doron and Snydman, 2015). In a study to assess the ability of a collection of lactobacilli to bind fibrinogen, only one strain (*L. salivarius* CCUG 47825) isolated from a case of septicemia, was found to strongly adhere to fibrinogen (Collins et al., 2012). Furthermore, this strain was found to aggregate human platelets at a level comparable to the human pathogen *S. aureus.* By sequencing the genome of CCUG 47825, candidate genes responsible for fibrinogen binding were identified. Complementing the genetic analysis with traditional molecular microbiological techniques enabled the identification of the novel fibrinogen receptor, CCUG_2371. Although only strain CCUG 47825 bound fibrinogen under laboratory conditions, homologues of the novel fibrinogen binding gene CCUG_2371 are widespread among *L. salivarius* strains, maintaining their potential to bind fibrinogen if expressed. This serves to highlight the fact that without a full genetic analysis of strains for human consumption, potential pathogenicity traits may go undetected (Collins et al., 2012).

The *L. salivarius* strains included in this study were very susceptible to most of the antimicrobials tested. In fact, their MICs were lower than the cut-offs established for lactobacilli to seven out of the eight antibiotics required for this species by the European Food Safety Authority (EFSA, 2012). The only exception was kanamycin. Intrinsic resistance of lactobacilli to kanamycin and other aminoglycosides (such as neomycin or streptomycin) has been repeatedly reported (Charteris et al. 1998; Danielsen and Wind 2003; Gueimonde et al., 2013), and this is thought to be a *L. salivarius* species-specific trait due to the lack of cytochrome-mediated transport of this class of antibiotics (Bryan and Kwan, 1981); the *L. salivarius* strains were also resistant to vancomycin but assessment of vancomycin sensitivity is not required by EFSA in the case of homofermentative lactobacilli (including *L. salivarius*) since they are intrinsically resistant to this antibiotic probably due to the presence of D-Ala-D-lactate in their peptidoglycan structure (Handwerger et al., 1994). As recognized by EFSA (2012), these types of intrinsic resistances do not represent a human health risk. Therefore, *L.*

salivarius V4II-90 and the other strains evaluated in this study can be considered as safe from this point of view.

Lactobacilli are among the Gram-positive bacteria with potential to produce biogenic amines and these substances can cause several toxicological problems and/or may act as potential precursors of carcinogenic nitrosamines (Bover-Cid and Holzapfel, 1999). The screened *L. salivarius* strains neither produced histamine, tyramine, putrescine or cadaverine nor harbored the gene determinants required for their biosynthesis. Additionally, they were unable to degrade gastric mucin in vitro.

Some studies have evaluated the potential of different lactic acid bacteria strains or their metabolites to inhibit the growth of *S. agalactiae* (Marsalková et al., 2004; Lee, 2005; Açikgöz et al., 2005; Zárate et al., 2006; Ermolenko et al., 2007; Bodaszewska et al., 2010; Tsapieva et al., 2011; Ruiz et al., 2012; De Gregorio, 2014). However, to our knowledge, this work includes the first human clinical trial focused on the rectal and vaginal eradication of GBS in pregnant women by using a probiotic strain. The criteria followed for the selection of the best candidate for such a target (*L. salivarius* V4II-90) allowed a notable reduction in the rate of GBS-colonized women and led to a sharp reduction in the use of antibiotics during the peripartum period. Hopefully, this strain will be applied in the next future to decrease GBS colonization during pregnancy, thus allowing a reduction in the use of IAP. In addition, it can be a very valuable tool to prevent GBS in the neonatal units or as a treatment adjunct in GBs infections.

VII. CONCLUSIONS/CONCLUSIONES

The conclusions derived from this PhD Thesis are the following:

Las conclusiones derivadas de esta Tesis son las siguientes:

FIRST. *Lactobacillus* was the dominant genus in the vaginal microbiota of both non-pregnant and pregnant fertile women that participate in the first objective of this PhD Thesis, while the rate of *Streptococcus agalactiae* colonization was 25 and 20%, respectively.

PRIMERA. *Lactobacillus* fue el género dominante en la microbiota vaginal de las mujeres no embarazadas y embarazadas que participaron en el primer objetivo de esta tesis, mientras que la tasa de colonización de *Streptococcus agalactiae* fue del 25 y 20%, respectivamente.

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SECOND. Among the 89 strains of lactobacilli isolated in this thesis, only 10 met the requirements initially established for their pre-selection as probiotics: (1) absence of *S. agalactiae*, *Gardnella vaginalis, Candida spp., Ureaplasma* spp. and *Mycoplasma spp* in the vaginal samples from which the lactobacilli were originally isolated; (2) Qualified Presumption of Safety (QPS) status of the *Lactobacillus* species; and (3) good growth and stability under laboratory conditions. The 10 preselected strains belonged to the same species: *Lactobacillus salivarius*.

SEGUNDA. Entre las 89 cepas de lactobacilos aisladas en esta tesis, únicamente 10 reunieron los requisitos que se habían establecido inicialmente para su preselección como probióticos: (1) ausencia de *S. agalactiae*, *Gardnerella vaginalis*, *Candida spp.*, *Ureaplasma* spp. y *Mycoplasma spp.* en las muestras vaginales de las que se habían aislado; (2) pertenecer a una especie con presunción cualificada de seguridad (estatus QPS); y (3) buen crecimiento y estabilidad en condiciones laboratoriales. Las 10 cepas preseleccionadas pertenecían a la misma especie: *Lactobacillus salivarius*.

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THIRD. Assessment of several probiotic properties, including specific antagonism against GBS, showed that *L. salivarius* V4II-90 was the strain with the highest potential for the eradication of GBS during pregnancy. This strain also fulfilled all the requirements of the European Food Safety Authority to be considered as a safe strain for human use, including the evaluation of its *in vivo* toxicity in a rat model.

TERCERA. La evaluación de varias propiedades probióticas, incluyendo el antagonismo específico frente a *S. agalactiae*, mostró que *L. salivarius* V4II-90 era la

cepa con un mayor potencial para la erradicación de *S. agalactiae* durante el embarazo. Esta cepa también cumplía todos los requisitos exigidos por la Autoridad Europea de Seguridad Alimentaria para ser considerada como una cepa segura para uso humano, incluyendo la evaluación de su toxicidad *in vivo* en un modelo rata.

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FOURTH. The administration of *L. salivarius* V4II-90 to GBS-positive pregnant women (10^9 cfu orally, once a day from week 26 to week 38 of pregnancy) led to the eradication of this species in the rectal and vaginal samples of 72% of the recruited women. No adverse effects were recorded from any of the women who ingested the probiotic strain.

CUARTA. La administración de *L. salivarius* V4II-90 a mujeres embarazadas positivas a *S. agalactiae* (10^9 ufc por vía oral, una vez al día desde las semana 26 hasta la 38 de embarazo) condujo a la erradicación de dicha especie en las muestras rectales y vaginales del 72% de las mujeres participantes. No se registraron efectos adversos en ninguna de las mujeres que ingirieron la cepa probiótica.

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GENERAL CONCLUSION/CONCLUSIÓN GENERAL

The administration of *L. salivarius* V4II-90 to GBS-positive pregnant women is a safe and successful strategy to significantly decrease the rates of GBS colonization during pregnancy and, therefore, to reduce the exposure of pregnant women and their infants to intrapartum prophylaxis.

La administración de *L. salivarius* V4II-90 a mujeres embarazadas positivas a *S. agalactiae* es una estrategia segura y eficaz para disminuir significativamente las tasas de colonización durante el embarazo y, por lo tanto, para reducir la exposición de las mujeres embarazadas y sus hijos a la profilaxis intraparto.

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